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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: C12N 15/62, 15/13, C07K 19/00, 16/30, A61K 47/42, 39/395, G01N 33/577, 33/574, C07K 16/00, 16/46 // 14/21	A1	(11) International Publication Number: WO 96/13594 (43) International Publication Date: 9 May 1996 (09.05.96)								
(21) International Application Number: PCT/US95/13811 (22) International Filing Date: 26 October 1995 (26.10.95) (30) Priority Data: <table border="0"> <tr> <td>08/331,396</td> <td>28 October 1994 (28.10.94)</td> <td>US</td> </tr> <tr> <td>08/331,397</td> <td>28 October 1994 (28.10.94)</td> <td>US</td> </tr> <tr> <td>08/331,398</td> <td>28 October 1995 (28.10.95)</td> <td>US</td> </tr> </table> (71) Applicant: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Box OTT, Bethesda, MD 20892 (US).	08/331,396	28 October 1994 (28.10.94)	US	08/331,397	28 October 1994 (28.10.94)	US	08/331,398	28 October 1995 (28.10.95)	US	(72) Inventors: PASTAN, Ira; 11710 Beall Mountain Road, Potomac, MD 20854 (US). BENHAR, Itai; 341 Congressional Lane, Rockville, MD 20852 (US). PADLAN, Eduardo, A.; 4006 Simms Drive, Kensington, MD 20895 (US). JUNG, Sun-Hee; 259 Congressional Lane #206, Rockville, MD 20852 (US). LEE, Byungkook; 10711 Sandy Landing Road, Potomac, MD 20854 (US). WILLINGHAM, Mark; 108 Kimberwicke Drive, Summerville, SC 29483 (US). FITZGERALD, David; 1202 Azalea Drive, Rockville, MD 20850 (US). BRINKMANN, Ulrich; 4404 Everett Street, Kensington, MD 20895 (US). PAI, Lee; 34 Tivoli Lake Street, Silver Spring, MD 20906 (US). (74) Agents: WEBER, Ellen, Lauer et al.; Townsend and Townsend and Crew, Stuart Street Tower, One Market, San Francisco, CA 94105-1492 (US). (81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
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TUMOR-SPECIFIC ANTIBODY FRAGMENTS, FUSION PROTEINS, AND USES THEREOF

The present application is a continuation-in-part of U.S. patent applications serial number 08/331,396, 08/331,397, and 08/331,398 all of which were filed on October 28, 1994 and all of which are continuations in part of U.S. patent application 07/767,331, filed on September 30, 1991 which is a continuation-in-part of U.S. patent application serial number 07/596,289 filed on October 12, 1990, all of which are hereby incorporated herein by reference for all purposes.

BACKGROUND OF THE INVENTION

The subject invention relates to tumor-specific recombinant antibody fragments, to molecules incorporating such fragments such as immunotoxins and to uses thereof. Exemplary embodiments of the invention include immunotoxins comprising *Pseudomonas* exotoxins fused to the Fv regions of monoclonal antibodies B1, B3, and B5 which have tumor specificity and which may be used in the treatment of mammalian cancer.

Monoclonal antibodies B1, B3, and B5 are recently isolated murine antibodies directed against a carbohydrate antigen in the Lewis^x (Le^x) family (Pastan *et al. Cancer Res.*, 51: 3781-3787 (1991)). The Le^x antigens are found on the surface of many mucinous carcinomas of the colon, stomach, ovaries, breast, lung as well as some epidermal carcinomas. Because they react with only a limited number of normal tissues, these antibodies are ideal candidates for use in the treatment and diagnosis of cancer.

In order to create a cytotoxic agent that specifically attacks cancer cells, an antibody or its fragments may be used as the targeting moiety of an immunotoxin. In such immunotoxins, the targeting moiety typically replaces the cell binding domain of a cytotoxin molecule (*e.g.* domain I of *Pseudomonas* exotoxin (PE) or the B chain of Diphtheria toxin) and acts to specifically direct the cytotoxin to its target cell (as determined by the specificity of the targeting moiety). As a result, only cells which are recognized by the targeting moiety are efficiently killed and cells which are not

recognized are spared (for a review *see* Brinkmann and Pastan, *Biochem. Biophys. Acta.*, 1198: 27-45 (1994)).

Immunotoxins were first made by chemically coupling antibodies to cytotoxic molecules. Thus, for example, monoclonal antibody B3 has been chemically coupled to at least two different forms of *Pseudomonas* exotoxin (PE) (U.S. Patent 4,545,985). One of these is the full length toxin (PE) and the other a truncated derivative (PE40) (Kondo *et al.*, *J. Biol. Chem.*, 263: 9470-75 (1988) and Pai *et al.*, *supra*). Both of these immunotoxins have been shown to be selectively cytotoxic to tumor cells that contain the B3 antigen on their surface, and these immunotoxins have been shown to cause complete tumor regression in mice bearing human tumor xenografts (Pai *et al.*, *Proc. Natl. Acad. Sci. USA*, 88: 3358-62 (1991)).

Although chemically coupled immunotoxins are useful they have several undesirable properties. For example, the chemical modifications can change the antibody and affect its binding to the antigen. Furthermore, the purified immunotoxins are a heterogeneous mixture of antibody-toxin molecules connected to each other via different positions on the antibody and the toxin. Thus, *Pseudomonas* exotoxin, for example, can be coupled either to the light- or heavy-chain of the antibody and to different positions on each of these chains.

To overcome the limitations of chemically conjugated immunotoxins, chimeric immunotoxins have been made as recombinant, single chain, antibody-toxin fusion proteins. It has been shown that certain single chain antigen binding proteins made from the Fv portions of the heavy and light chain of antibodies held together by a polypeptide linker can have the same binding properties as their full length two chain counterparts (Bird *et al.*, *Science*, 242: 423-26 (1988) and Huston *et al.*, *Proc. Natl. Acad. Sci. USA*, 85: 5879-83 (1988)). It has also been shown that, in some cases, fusion proteins composed of single chain antibodies linked to toxins may retain the binding capacity of the single chain antibody as well as the activity of the toxin (Chaudhary *et al.*, *Nature*, 339: 394-97 (1989); Batra *et al.*, *J. Biol. Chem.*, 265: 15198-15202 (1990); Batra *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 8545-8549 (1989); Chaudhary *et al.*, *Proc. Natl. Acad. Sci. USA* 87: 1066-1070 (1990)).

Receptor proteins have often been used as immunotoxin targets because they are cell surface proteins which are often overexpressed in various cancers (Brinkmann and Pastan, *Biochem. Biophys. Acta.*, 1198: 27-45 (1994)) and thus provide

cancer-specific targets. For example, single chain immunotoxins have been made consisting of the Fv domain of an antibody directed at the interleukin 2 receptor (Chaudhary *et al.*, *Nature*, 339: 394-97 (1989) and Batra *et al.*, *J. Biol. Chem.* 265: 15198-15202 (1990)) or at the transferrin receptor (Batra *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 8545-49 (1989)) fused to truncated forms of PE or diphtheria toxin (Chaudhary *et al.*, *Proc. Natl. Acad. Sci. USA* 87:9491-94 (1990)). Although receptor proteins are overexpressed on many cancers, they may still be present on healthy cells and therefore often do not provide the defined cancer specificity desired for an immunotoxin.

Since the number of antibodies that react preferentially with carcinomas is limited, the identification and characterization of additional "cancer specific" antibodies that would react with all or most of the cells in a tumor and with relatively few normal cells and tissues is desirable. In addition, recombinant immunotoxins are known to degrade over time both *in vitro* and *in vivo*. It would be desirable to obtain immunotoxins that show a reduced rate of degradation and therefore require less frequent administration. Finally, with repeated use, murine antibodies and fusion proteins containing murine antibodies, like any other foreign protein, may ultimately prove immunogenic and invoke an immune response in the treated organism. It would be desirable to produce targeting moieties and immunotoxins having reduced antigenic potential. As will be explained herein, these advantages and others are provided by the present invention.

SUMMARY OF THE INVENTION

The present invention provides for recombinant single chain antibodies and fusion proteins such as immunotoxins employing these antibodies. In particular, this invention provides for recombinantly produced antibodies comprising the variable light and heavy (Fv) chain regions of antibodies that have the binding specificity of monoclonal antibodies B1, B3, or B5. These antibodies provide carcinoma-specific targeting moieties suitable for use in cytotoxic fusion proteins.

In one embodiment, this invention provides for single-chain antibodies comprising an Fv region of both the variable light (V_L) and variable heavy (V_H) chain regions of an antibody where the single-chain antibody has the binding specificity of monoclonal antibodies B1, B3, or B5. Particularly preferred are single chain antibodies B1(Fv), B3(Fv), and B5(Fv).

In another embodiment, this invention provides for single-chain antibodies comprising an Fv region in which position 95 of V_H is mutated to a serine when it is not normally a serine. The single-chain antibodies may be carbohydrate-binding antibodies and more preferably are Le^Y-binding antibodies having position 95 of V_H mutated to a serine. In most cases position 95 will be a tyrosine before mutation. Thus a particularly preferred antibody is B5(Fv): Y95S, described herein.

This invention also provides for single-chain B3 antibodies having various mutations that increase the stability of the antibody. Particularly preferred mutations are in the V_L chain and include a mutation of methionine to leucine at position 4 (B3(Fv): M4L, or a mutation of serine to threonine at position 7 (B3(Fv): S7T) or the combination of both mutations (B3(Fv): M4L S7T).

In another embodiment, this invention provides for chimeric single-chain antibodies comprising a variable heavy chain of a first antibody and a variable light chain of a second antibody where the first and second antibody are different antibodies and the heavy and light chain are recombinantly fused to form a single-chain antibody which specifically binds a Lewis^Y carbohydrate antigen. In a particularly preferred embodiment, the single-chain antibody has the binding specificity of monoclonal antibody B1, B3, or B5. In one embodiment, the first antibody is B1, B3, or B5. In another embodiment, the second antibody is B1, B3, or B5. Particularly preferred single chain antibodies include B5V_H-B3V_L and B3V_H-B5V_L.

This invention also provides for recombinantly produced humanized single-chain antibodies comprising humanized variable light and heavy (Fv) regions of antibodies that have the binding specificity of monoclonal antibody B1, B3 or B5. These antibodies provide carcinoma-specific targeting moieties suitable for use in cytotoxic fusion proteins. Particularly preferred are humanized single-chain Fv regions of B1, B3 or B5.

In one embodiment, the single-chain antibody is a humanized B3(Fv). Particularly preferred is an antibody comprising a humanized variable heavy chain, more specifically a humanized variable heavy chain having the amino acid sequence designated HumB3V_H in Figure 11. Another preferred variant is an antibody comprising a humanized variable light chain, more specifically a humanized variable light chain having the amino acid sequence designated HumB3V_L in Figure 11. Yet another preferred humanized antibody is one comprising both a humanized variable light chain and a

humanized variable heavy chain. Particularly preferred is an antibody comprising a humanized variable heavy chain having the amino acid sequence designated HumB3V_H in Figure 11 and a humanized variable light chain having the amino acid sequence designated HumB3V_L in Figure 11. Still yet another preferred humanized antibody is one comprising a humanized variable heavy chain having the amino acid sequence designated HumB3V_H in Figure 11 and a humanized variable light chain having the amino acid sequence designated HumB3V_L in Figure 11 in which the serine at the position designated as 82b in Figure 11, is replaced with arginine.

In any of the single chain antibodies described above, the variable heavy chain region and the variable light chain region may be joined by a linker. One particularly preferred linker is (Gly₄Ser)₃.

This invention also provides for single-chain fusion proteins incorporating any of the above-described single-chain antibodies. The fusion proteins comprise the single chain antibodies recombinantly fused to an effector molecule. The effector molecule may be a cytotoxin such as *Pseudomonas* exotoxin and more preferably is either PE38, PE40, PE38KDEL, or PE38REDL.

In a particularly preferred embodiment, the Fv region is a B1(Fv), a B3(Fv), or a B5(Fv) region or any of the modified B1(Fv), B3(Fv) or B5(Fv) regions described above. Thus, preferred fusion proteins include B3(Fv)-PE38, B3(Fv)-PE40, B3(Fv)-PE38KDEL, B3(Fv)-PE38REDL, B1(Fv)-PE38, B1(Fv)-PE40, B1(Fv)-PE38KDEL, B1(Fv)-PE38REDL, B5(Fv)-PE38, B5(Fv)-PE40, B5(Fv)-PE38KDEL, B5(Fv)-PE38REDL, B3(Fv): S7T-PE38, B3(Fv): S7T-PE40, B3(Fv): S7T-PE38KDEL, B3(Fv): S7T-PE38REDL, B3(Fv): M4L-PE38, B3(Fv): M4L-PE40, B3(Fv): M4L-PE38KDEL, B3(Fv): M4L-PE38REDL, B3(Fv): M4L S7T-PE38, B3(Fv): M4L S7T-PE38KDEL, B3(Fv): M4L S7T-PE38REDL, B5V_H-B3V_L-PE38, B5V_H-B3V_L-PE40, B5V_H-B3V_L-PE38KDEL, B5V_H-B3V_L-PE38REDL, B3V_H-B5V_L-PE38, B3V_H-B5V_L-PE40, B3V_H-B5V_L-PE38KDEL, and B3V_H-B5V_L-PE38REDL, HUMB3(Fv)-PE38, HUMB3(Fv)-PE40, HUMB3(Fv)-PE38KDEL, HUMB3(Fv)-PE38REDL, B5(Fv): Y95S-PE38, B5(Fv): Y95S-PE40, B5(Fv): Y95S-PE38KDEL and B5(Fv): Y95S-PE38REDL.

The fusion proteins may also include a linker between the variable heavy (V_H) and the variable light (V_L) chain regions of the Fv fragment. One preferred linker is the peptide linker (Gly₄Ser)₃. The fusion proteins may also include a connector

between the Fv region and the effector molecule. A particular preferred connector is SGGPEGGS.

5 All of the embodiments described above are recombinantly expressed as single chain fusion proteins. Thus, this invention also provides for recombinant DNA molecules that encode any of the above-described single-chain antibody Fv regions and fusion proteins.

10 In another embodiment this invention provides for a pharmaceutical composition comprising any of the single-chain fusion proteins described above in a concentration sufficient to inhibit tumor cell growth together with a pharmaceutically acceptable carrier.

This invention similarly provides for a method of killing or inhibiting the growth of cells bearing a Lewis^Y antigen in a patient. The method includes the steps of administering to the patient a pharmaceutical composition comprising any of the fusion proteins described above in an amount sufficient to kill or inhibit the growth of the cells.

15 This invention also provides for a method of detecting the presence or absence of a cell bearing a Lewis^Y carbohydrate antigen in a patient, the method comprising the steps of removing a tissue or fluid sample from the patient, adding any of the single-chain antibodies described above to the sample, and detecting for the presence or absence of a binding complex between the antibody and the antigen.

20 In yet another embodiment, this invention provides for a method of improving the binding affinity of antibodies that lack a serine at position 95 of the V_H region. The method includes the step of replacing the amino acid at position 95 of V_H with a serine. The antibody is preferably a carbohydrate-binding antibody and even more preferably an anti-Le^Y antibody. The amino acid to be mutated at position 95 of V_H will, in most cases, be a tyrosine.

BRIEF DESCRIPTION OF THE DRAWINGS

30 Figure 1a illustrates the strategy for the cloning of the heavy and light chain Fv genes of monoclonal antibody B3 and the construction of expression vectors (e.g., plasmids) for the expression of B3(Fv) immunotoxins. The cloning strategy is a variation of that previously described (Chaudhary *et al.*, *Proc. Natl. Acad. Sci. USA*, 87: 1066-70 (1990)). The plasmid pVC38H, which is used as a vector for construction of immunotoxins from heavy and light chain Fv regions, contains an NdeI and a HindIII

recognition sequence preceding the PE40 gene (Chaudhary *et al.*, *supra* (1990)). (*) indicates a PCR-generated mutation and was repaired by site directed mutagenesis; (L) indicates the region encoding the (Gly₄Ser)₃ linker (Sequence ID No. 32) which serves to join heavy and light chains of the immunotoxin.

5 Figure 1b shows the construction LMB7, the immunotoxin B3(Fv)-PE38 with a "C3 connector" between the Fv region and the PE38 cytotoxin.

Figure 2 shows the nucleotide sequences encoding the heavy and light chain Fv region of monoclonal antibody B3 (Sequence ID No. 33). (a) The heavy chain Fv coding region extends from position 30 to 383, the light chain Fv gene from position 433 to 767 and the linker from 384 to 432. The deduced amino acid sequence is shown
10 in plain letters (Sequence ID No. 34); below in italic letters is the protein sequence determined by Edman sequencing of the antibody. The first amino acid encoded by the cloned heavy chain Fv gene is Asp instead of Glu due to the oligonucleotide primer used at position 456-465. This is the region where the PCR cloning artifact was repaired.
15 This sequence encodes the same amino acids as the original B3 light chain gene but uses other codons. Homology comparisons to the known nucleotide sequence of PACT Ig kappa chain (Taub *et al.*, *J. Biol. Chem.*, 264: 59-65 (1989)) which is most homologous to the B3 light chain indicates that the original sequence was most probably CTCTCCCTG (Sequence ID No. 37) instead of TTGAGTTTA (Sequence ID No 38).
20 Thus the natural B3 light chain gene has a sequence repetition 5-(CCAGTCT[CC]ACTCTCC)-3' (Sequence ID No. 39) between positions 445 and 465 which is responsible for the incorrect primer annealing in PCR. (b) Sequence at the 3'-end of the light chain for expression of the single chain B3(Fv) alone (Sequence ID No. 35 and amino acid sequence Sequence ID No. 36). (SD) - Shine Dalgarno consensus
25 sequence; (*) - translation stop signal. (Term) transcription terminator.

Figure 3(a) represents the toxicity of B3'(Fv)-PE38KDEL on different cell lines. Cytotoxicity assays were performed as described in Example 7. (b): Inhibition of the cytotoxicity of B3(Fv)-PE38KDEL by monoclonal antibody B3. Competition by monoclonal antibody B3 was performed on A431 cells as described in Example 7.

30 Figure 4 shows the ADP-ribosylation and cytotoxic activities of B1(Fv)-PE38, B3(Fv)-PE38, B5(Fv)-PE38 recombinant immunotoxins. (A) ADP-ribosylation activity was determined by the incorporation on ¹⁴C-NAD into acid-precipitable material using elongation factor 2 enriched wheat-germ extract (Collier and Kandel, 1971). (B)



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which were diluted in PBS +0.2% BSA following incubation at 37°C. ³H-Leucine was added 20 hours after addition of immunotoxins.

Figure 11 illustrates the humanization of B3(Fv). Alignment of the amino acid sequences of (A) B3 V_H, 56P1'CL V_H and HUMB3V_H (Sequence ID Nos. 45, 46 and 47) (B) B3 V_L, GM607 V_L, and HUMB3V_L (Sequence ID Nos. 48, 49 and 50). B3 amino acids that differ from the residues of the corresponding position of the human antibody are indicated by vertical lines above the sequence. Inter-domain residues that were not humanized are indicated by asterisks below the sequence. Heavy chain residue 82b is underlined. Numbers above the sequence indicate the positions of residues that were humanized.

Figure 12 illustrates the plasmids utilized for expression of humanized B3(Fv)-PE38 immunotoxins. Single-stranded uracil-containing pULI7 DNA (A) encoding wild type B3(Fv)-PE38 was the template for the mutagenesis according to the method of Kunkel, *Proc. Natl. Acad. Sci. USA* 82,488-492 (1985). Single stranded uracil-containing pB3V_H-HUMV_L-PE38 DNA (C) was the template for the generation of pHUMB3(Fv)-PE38.

Figure 13 shows the ADP-ribosylation activity, cytotoxicity, and antigen binding of B3(Fv)PE38 and of the humanized variants. (A) ADP-ribosylation activity was determined by the incorporation on ¹⁴C-NAD into acid precipitable material using elongation factor 2 enriched wheat-germ extract. (B) Cytotoxicity towards A431 cells was measured by the inhibition of incorporation of [³H]-leucine into cell protein. (C) Antigen binding was estimated by competition of [¹²⁵I]-B3 IgG binding to A431 cells at 4°C with each immunotoxin.

Figure 14 shows the reactivity of pooled monkey anti- B3(Fv)-PE38 sera to B3(Fv)PE38 and humanized variants. B3(Fv)-PE38, B3HUMVH-HUMVL-PE38 and HUMB3(Fv)-PE38 were immobilized on a 96-well microtiter plate. Sera that were preincubated with PE38 as a competitor at a molar ratio of 1000 to 1 over the immobilized proteins were added in an equal volume at a dilution of 1:50. Percent reactivity was calculated by setting the mean reactivity with B3(Fv)-PE38 obtained from four independent experiments to 100% and adjusting the relative reactivities with the humanized variant accordingly.

Figure 15 provides the nucleotide and deduced amino acid sequences of B1 heavy (A) and light (B) chains. Underlined nucleotide sequences correspond (at the 5'

end) or are complementary (at the 3' end) to the PCR primers which were used to PCR amplify the fragment. The amino acid sequence is in single-letter code; below is the amino acid sequence determined by Edman sequencing shown in *italics*. CDRs are underlined, and constant region amino acids are struck through.

5 Figure 16 provides the nucleotide and deduced amino acid sequence of B5 heavy chain (A) and the variable region and the beginning of the constant region of the light (B) chain. Other details are as in Figure 15. The struck-through carboxyl-terminal amino acids in the heavy chain correspond to the beginning of the (Gly₄Ser)₃ linker used to connect the V_H and the V_L in the single chain configuration.

10 Figure 17 provides the amino acid (peptide) sequence of the B3 single chain Fv. The figure provides the sequences for the V_H region, the linker and the V_L region respectively. CDRs are in parentheses.

 Figure 18 provides the amino acid (peptide) sequence of the humanized B3 single-chain Fv. CDRs are in parentheses.

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DETAILED DESCRIPTION OF THE INVENTION

Definitions

Abbreviations used here for the twenty naturally occurring amino acids, the five naturally occurring nucleic acids and the eleven nucleic acid degeneracies (wobbles) follow conventional usage. In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction. In the nucleic acid notation used herein, the left-hand direction is the 5' direction and the right-hand direction is the 3' direction.

25 The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a manner similar to naturally occurring nucleotides.

30 The phrase "nucleic acid encoding" or "nucleic acid sequence encoding" refers to a nucleic acid which directs the expression of a specific protein or peptide. The nucleic acid sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The nucleic acid sequences include both full length nucleic acid sequences as well as shorter sequences derived from the full length sequences. It is understood that a particular nucleic acid sequence

includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell. The nucleic acid includes both the sense and antisense strands as either individual single strands or in the duplex form.

5 The terms "isolated" or "substantially purified", when referring to recombinantly produced proteins, means a chemical composition which is essentially free of other cellular components. Such a composition is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel
10 electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. Generally, a substantially purified or isolated protein will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein is purified to represent greater than 90% of all macromolecular species present. More preferably the
15 protein is purified to greater than 95%, and most preferably the protein is purified to essential homogeneity, wherein other macromolecular species are not detected by conventional techniques.

 The term "labeled antibody" as used herein refers to an antibody bound to a label such that detection of the presence of the label (*e.g.* as bound to a biological
20 sample) indicates the presence of the antibody.

 Cytotoxin refers to a molecule that when contacted with a cell brings about the death of that cell.

 The phrase "binding specificity", "specifically binds to an antibody" or "specifically immunoreactive with," when referring to a protein or carbohydrate, refers
25 to a binding reaction which is determinative of the presence of the protein or carbohydrate in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein or carbohydrate and do not bind in a significant amount to other proteins or carbohydrates present in the sample. Specific binding to an antibody under
30 such conditions may require an antibody that is selected for its specificity for a particular protein or carbohydrate. For example, antibodies raised to the Le^x antigens may be selected to provide antibodies that are specifically immunoreactive with Le^x proteins and not with other proteins. A variety of immunoassay formats may be used to select

antibodies specifically immunoreactive with a particular protein or carbohydrate. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein or carbohydrate. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, 5 for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

The terms "recombinant DNA," "recombinant nucleic acid" or "recombinantly produced DNA" refer to DNA which has been isolated from its native or endogenous source and modified either chemically or enzymatically by adding, deleting 10 or altering naturally-occurring flanking or internal nucleotides. Flanking nucleotides are those nucleotides which are either upstream or downstream from the described sequence or sub-sequence of nucleotides, while internal nucleotides are those nucleotides which occur within the described sequence or subsequence.

The terms "recombinant protein" or "recombinantly produced protein" 15 refer to a peptide or protein produced using non-native cells that do not have an endogenous copy of DNA able to express the protein. The cells produce the protein because they have been genetically altered by the introduction of the appropriate nucleic acid sequence. The recombinant protein will not be found in association with proteins and other subcellular components normally associated with the cells producing the 20 protein.

Mutations in proteins are designated by nomenclature consisting of the peptide sequence in which the mutation occurs, a representation of the non-mutated amino acid, followed by its position, followed by the representation of the mutated amino acid. Thus, for example, a mutation designated B3(Fv)_{V_L} S7T is a mutation from serine 25 (S) to threonine (T) at position 7 of the V_L chain of B3(Fv).

Single Chain Antibodies

This invention relates to recombinantly produced single chain antibodies. In particular, this invention provides for recombinant single chain antibodies that may be 30 joined to one or more effect r molecules and, because of their ability to specifically bind to a particular preselected target molecule, these antibodies are useful as targeting moieties which serve to direct the joined effector molecules or compositions to a cell or tissue bearing the preselected target molecule.

As used herein, the term "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

The basic immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies may exist as intact immunoglobulins, or as modifications in a variety of forms including, for example, an Fv fragment containing only the light and heavy chain variable regions, a Fab or (Fab)₂ fragment containing the variable regions and parts of the constant regions, a single-chain antibody (Bird *et al.*, *Science* 242: 424-426 (1988); Huston *et al.*, *Proc. Natl. Acad. Sci. USA* 85: 5879-5883 (1988) both incorporated by reference herein), and the like. The antibody may be of animal (especially mouse or rat) or human origin or may be chimeric (Morrison *et al.*, *Proc Natl. Acad. Sci. USA* 81, 6851-6855 (1984) both incorporated by reference herein) or humanized (Jones *et al.*, *Nature* 321, 522-525 (1986), and published UK patent application #8707252, both incorporated by reference herein). As used herein the term "antibody" includes these various forms. Using the guidelines provided herein and those methods well known to those skilled in the art which are described in the references cited above and in such publications as Harlow & Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, (1988) the antibodies of the present invention can be readily made.

The term "Fv" region as used herein refers to a single chain antibody Fv region containing a variable heavy (V_H) and a variable light (V_L) chain. The heavy and light chain may be derived from the same antibody or different antibodies thereby producing a chimeric Fv region.

The term "effector molecule" or "effector composition" as used herein refer to agents having a particular biological activity which is to be directed to a particular target molecule or a cell bearing a target molecule. One of skill in the art will appreciate that effector molecules may include various drugs such as vinblastine, daunomycin and the like, cytotoxins such as native or modified *Pseudomonas* exotoxin or Diphtheria toxin, encapsulating agents (e.g., liposomes) which themselves contain pharmacological compositions, radioactive agents such as ^{125}I , ^{131}Cs , ^{32}P , ^{14}C , ^3H , and ^{35}S , target moieties and ligands.

As used herein "ligands" are molecules capable of reacting with or otherwise recognizing and specifically binding a "target" molecule. Ligands and their respective target molecules represent paired species. Typical paired species include, but are not limited to, enzyme/substrate, receptor/agonist, antibody/antigen, and lectin/carbohydrate. The binding between a ligand and its target may be mediated by covalent or non-covalent interactions or a combination of covalent and non-covalent interactions. When the interaction of the two species produces a non-covalently bound complex, the binding which occurs is typically electrostatic, hydrogen-bonding, or the result of hydrophilic/lipophilic interactions. Accordingly, "specific binding" occurs between a ligand and its target molecule where there is interaction between the two which produces a bound complex having the characteristics of an antibody/antigen or enzyme/substrate interaction. Specifically, examples of ligands include, but are not limited to antibodies, lymphokines, cytokines, receptor proteins such as CD4 and CD8, solubilized receptor proteins such as soluble CD4, hormones, growth factors, and the like which specifically bind desired target cells.

The choice of the particular effector molecule or composition depends on the particular target molecule or cell and the biological effect it is desired to evoke. Thus, for example, the effector molecule may be a cytotoxin where it is desired to bring about death of a particular target cell. Conversely, where it is merely desired to invoke a non-lethal biological response, the effector molecule may be a conjugated non-lethal pharmacological agent or a liposome containing a non-lethal pharmacological agent.

In a particularly preferred embodiment, the antibodies may be joined to an effector molecule that is a drug or to a cytotoxin to form an immunotoxin capable of selectively killing particular target cells. Numerous cytotoxic compounds are known to those of skill in the art and include, but are not limited to, ricin, abrin, *Pseudomonas*

exotoxin (PE), Diphtheria toxin (DT), and the like. Preferred toxins are PE or DT. Native PE and DT are highly toxic compounds that typically bring about death through liver toxicity. PE and DT, however, can be modified into a form for use as an immunotoxin by removing the native targeting component of the toxin (*e.g.* domain Ia of PE and the B chain of DT) and replacing it with a different antibody targeting moiety.

The term "*Pseudomonas* exotoxin" (PE) as used herein refers to a full-length native (naturally occurring) PE or a PE that has been modified. Such modifications may include, but are not limited to, elimination of domain Ia, various amino acid deletions in domains II and III, single amino acid substitutions (*e.g.*, replacing Lys with Gln at positions 590 and 606), and the addition of one or more sequences at the carboxyl terminus such as KDEL (Seq. ID No: 51) and REDL (Seq. ID No: 52). See Siegall *et al.*, *J. Biol. Chem.* 264: 14256-14261 (1989). Thus, for example, PE38 refers to a truncated *Pseudomonas* exotoxin composed of amino acids 253-364 and 381-613 (*see* commonly assigned U.S. Patent Application Serial Number 07/901,709 filed June 18, 1992 incorporated herein by reference. The native C-terminus of PE, REDLK (residues 609-613, Seq ID No: 53), may be replaced with the sequence KDEL, REDL, and Lys-590 and Lys-606 may be each mutated to Gln (*see* commonly assigned U.S. Patent Application Serial Number 07/522,563 filed May 14, 1990, incorporated herein by reference).

The term "Diphtheria toxin" (DT) as used herein refers to full length native DT or to a DT that has been modified. Modifications typically include removal of the targeting domain in the B chain and, more specifically, involve truncations of the carboxyl region of the B chain.

The recombinant single chain antibodies of the present invention may be fused to, or otherwise bound to the effector molecule or composition by any method known and available to those in the art. The two components may be chemically bonded together by any of a variety of well-known chemical procedures. For example, the linkage may be by way of heterobifunctional cross-linkers, *e.g.* SPDP, carbodiimide, glutaraldehyde, or the like. Production of various immunotoxins is well-known within the art and can be found, for example in "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet," Thorpe *et al.*, *Monoclonal Antibodies in Clinical Medicine*, Academic Press, pp. 168-190 (1982) and Waldmann, *Science*, 252: 1657 (1991), both of which are incorporated by reference. To use the recombinant PE molecules with an

antibody, a form of the PE molecule with cysteine at amino acid position 287 is preferred to couple the toxin to the antibody or other ligand through the thiol moiety of cysteine.

5 In a preferred embodiment, the antibodies of this invention may also be fused to a protein effector molecule by recombinant means such as through the use of recombinant DNA techniques to produce a nucleic acid which encodes both the antibody and the effector molecule and expressing the DNA sequence in a host cell such as *E. coli*. The DNA encoding the chimeric protein may be cloned in cDNA or in genomic form by any cloning procedure known to those skilled in the art. See for example
10 Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor laboratory, (1989), which is herein incorporated by reference.

As indicated above, in addition to cytotoxins, the single chain antibodies of the present invention may be fused or chemically conjugated to a wide variety of effector molecules. Thus, for example, the antibody may be conjugated or fused to bacterial or
15 plant toxins, or to other effector agents to treat or diagnose human cancer. For example, radionuclides conjugated to antibodies that bind to tumors can produce cell killing based on the high local concentration of radiation. Chemotherapeutic drugs, for example, vinblastine or daunomycin, can be coupled to the antibodies and delivered at high concentration to cells that react with the antibodies. Similarly, the antibodies of this
20 invention may be utilized to specifically target a vehicle that encapsulates a therapeutic agent. For example, the antibodies may be conjugated to a liposome which itself carries a drug (*e.g.* doxorubicin) and thereby specifically targets the liposome to a specific tissue or cell. Alternatively, the antibodies may be recombinantly fused to a membrane-inserting protein and thereby incorporated into the liposome for delivery of therapeutic
25 agents.

Fusion or conjugation of the antibodies of this invention to various labels produces a highly specific detectable marker that may be used to detect the presence or absence of cells or tissues bearing the particular molecule to which the antibody is detected. Alternatively, the antibodies may be chemically conjugated or fused to an
30 effector molecule that is another specific binding moiety, *e.g.* a ligand such as those described above. In this form the composition will act as a highly specific bifunctional linker. This linker may act to bind and enhance the interaction between cells or cellular components to which the fusion protein binds. Thus, for example, where the fusion

protein is a growth factor joined to an antibody or antibody fragment (*e.g.* an Fv fragment of an antibody), the antibody may specifically bind antigen positive cancer cells while the growth factor binds receptors (*e.g.*, IL2 or IL4 receptors) on the surface of immune cells. The fusion protein may thus act to enhance and direct an immune response toward target cancer cells.

One of skill in the art will appreciate that the antibodies of the present invention may also be utilized as multiple targeting moieties. Thus this invention also provides for compositions in which two or more antibodies are bound to a single effector molecule. Where the effector molecule is a cytotoxin, the presence of two or more antibodies may increase specificity or avidity of binding of the immunotoxin. Conversely, multiple effector molecules may be fused or otherwise joined to a single antibody. Compositions of this nature may provide two or more kinds of biological activity with a single targeting moiety.

In a particularly preferred embodiment, the antibodies of this invention are antibodies that specifically bind Lewis^Y (Le^Y) carbohydrates (Le^Y carbohydrate antigens). As used herein, the Le^Y carbohydrate antigens include natural or synthetic Le^Y carbohydrates or fragments thereof that contain epitopes recognizable by Le^Y binding antibodies. Also included are carbohydrates, glycoproteins and other glycoconjugates which contain or mimic the Le^Y carbohydrate or epitopes contained within the Le^Y carbohydrate (*see*, Pastan *et al.*, *Cancer Res.*, 51: 3781-3787 (1991) and Hoess *et al.*, *Gene*, 128: 43-49 (1993)). Such mimics are known by their ability to specifically bind to known anti-Le^Y antibodies such as B1, B3, B5, BR64 and BR96 (Hellstrom *et al.*, *Cancer Res.*, 50:2183-90 (1990)), and the like.

Of the Le^Y binding antibodies, particularly preferred are antibodies having the tissue binding specificity of B1, B3 or B5. The term "tissue binding specificity" as used herein refers to the particular distribution of tissues to which an antibody binds and does not bind as determined by immunohistochemical analysis. Methods of determining tissue binding specificity as well as the binding specificities for B1, B3 and B5 are described in U.S. Patent 5,242,813 (*see*, especially Tables I, II and III) which is incorporated herein by reference.

The antibodies may be derived from the monoclonal antibodies designated B1, B3, and B5 (*see* U.S. Patent 5,242,813). These antibodies have been shown to specifically bind to Lewis^Y and Lewis^Y-related carbohydrate antigens that are typically

f und on various carcinomas including carcinomas of the breast, colon, cervix, and prostate.

The antibodies of this invention may be Fv regions comprising a variable light (V_L) and a variable heavy (V_H) chain. The light and heavy chains may be joined directly or through a linker. As used herein a linker refers to a molecule that is covalently linked to the light and heavy chain and provides enough spacing and flexibility between the two chains such that they are able to achieve a conformation in which they are capable of specifically binding the epitope to which they are directed. Protein linkers are particularly preferred as they may be expressed as an intrinsic component of the fusion protein.

Preparation of Antibody Fv Fragments

Single chain B1, B3 and B5 Fv regions may be cloned from the hybridoma cell lines B1, B3 and B5 which were deposited on October 10, 1990 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, where the deposits were granted the accession numbers ATCC HB 10572, HB 10573, and HB 10569, respectively. The deposits were made pursuant to the provisions of the Budapest Treaty.

The Fv regions may all be cloned using the same general strategy. Typically, for example, poly(A)⁺ RNA extracted from the hybridoma cells is reverse transcribed using random hexamers as primers. The V_H and V_L domains are amplified separately by two polymerase chain reactions (PCR[®]). Heavy chain sequences may be amplified using 5' end primers which are designed according to the amino-terminal protein sequences of the B1, B3 and B5 heavy chains respectively (Sequence Listing ID Nos. 19, 17, and 21 respectively) and 3' end primers according to consensus immunoglobulin constant region sequences (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*. 5th edition. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD (1991) incorporated by reference). Light chain Fv regions are amplified using 5' end primers designed according to the amino-terminal protein sequences of B1, B3 and B5 light chains respectively (Sequence ID Nos. 20, 18 and 22 respectively) and in combination with the primer C-kappa (Table 1 and Sequence ID No. 14). Suitable primers are specifically illustrated in Examples 1 and 2 although one of skill in the art would recognize that other suitable primers may be derived from the sequence listings provided herein.

The crude PCR products are subcloned into a suitable cloning vector. Clones containing the correct size insert by DNA restriction are identified. The nucleotide sequence of the heavy or light chain coding regions may then be determined from double stranded plasmid DNA using sequencing primers adjacent to the cloning site. Commercially available kits (e.g. the Sequenase™ kit, United States Biochemical Corp., Cleveland, Ohio, USA) may be used to facilitate sequencing the DNA.

Of course the sequencing steps are unnecessary given the sequence information disclosed in the present invention. One of skill will appreciate that utilizing the sequence information provided for the Fv regions of B1, B3, and B5 (Sequence ID Nos. 17-22), nucleic acids encoding these sequences may be obtained using a number of methods well known to those of skill in the art. Thus, DNA encoding the Fv regions may be prepared by any suitable method, including, for example, amplification techniques such as ligase chain reaction (LCR) (see Wu and Wallace, *Genomics*, 4: 560 (1989), Landegren, *et al.*, *Science*, 241: 1077 (1988) and Barringer, *et al.*, *Gene*, 89: 117 (1990)), transcription amplification (see Kwok, *et al.*, *Proc. Natl. Acad. Sci. USA*, 86: 1173 (1989)), and self-sustained sequence replication (see Guatelli, *et al.*, *Proc. Natl. Acad. Sci. USA*, 87: 1874 (1990)), cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.* *Meth. Enzymol.* 68: 90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.*, 22: 1859-1862 (1981); and the solid support method of U.S. Patent No. 4,458,066, all such references in this paragraph incorporated by reference herein.

Chemical synthesis produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. While it is possible to chemically synthesize an entire single chain Fv region, it is preferable to synthesize a number of shorter sequences (about 100 to 150 bases) that are later ligated together.

Alternatively, subsequences may be cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments may then be ligated to produce the desired DNA sequence.

Once the Fv variable light and heavy chain DNA is obtained, the sequences may be ligated together, either directly or through a DNA sequence encoding

a peptide linker, using techniques well known to those of skill in the art. In a preferred embodiment, heavy and light chain regions are connected by a flexible peptide linker (e.g. (Gly₄Ser)₃) which starts at the carboxyl end of the heavy chain Fv domain and ends at the amino terminus of the light chain Fv domain. The entire sequence encodes the Fv domain in the form of a single-chain antigen binding protein.

Preparation of Antibody Fusion Proteins

Once a DNA sequence has been identified that encodes an Fv region which, when expressed shows specific binding activity, fusion proteins comprising that Fv region may be prepared by methods known to one of skill in the art. The Fv region may be fused directly to the effector molecule (e.g. cytotoxin) or may be joined directly to the cytotoxin through a peptide connector. The peptide connector may be present simply to provide space between the targeting moiety and the effector molecule or to facilitate mobility between these regions to enable them to each attain their optimum conformation. The DNA sequence comprising the connector may also provide sequences (such as primer sites or restriction sites) to facilitate cloning or may preserve the reading frame between the sequence encoding the targeting moiety and the sequence encoding the effector molecule. The design of such connector peptides will be well known to those of skill in the art. However, one particularly preferred connector is the peptide SGGPEGGS (Sequence ID No. 44), designated herein as the C3 connector.

Methods of producing fusion proteins are well known to those of skill in the art. Thus, for example, Chaudhary *et al.*, *Nature*, 339: 394-97 (1989); Batra *et al.*, *J. Biol. Chem.* 265: 15198-15202 (1990); Batra *et al.*, *Proc. Natl. Acad. Sci. USA*, 86: 8545-8549 (1989); Chaudhary *et al.*, *Proc. Natl. Acad. Sci. USA*, 87: 1066-1070 (1990), all incorporated by reference, describe the preparation of various single chain antibody-toxin fusion proteins.

Generally producing immunotoxin fusion proteins involves separately preparing the Fv light and heavy chains and DNA encoding any other protein to which they will be fused and recombining the DNA sequences in a plasmid or other vector to form a construct encoding the particular desired fusion protein. However, a simpler approach involves inserting the DNA encoding the particular Fv region into a construct already encoding the desired second protein.

Thus, for example, DNA encoding B1(Fv), B3(Fv), B5(Fv) or chimeric Fv fusion proteins is most easily prepared by inserting the DNA encoding the B1, B3, B5 or chimeric Fv regions into constructs already containing DNA encoding the desired cytotoxin. The expression plasmid pVC38H contains the gene from the immunotoxin TGF α -PE40 under control of the T7 promoter, the T ϕ transcription terminator at the 3' end of the PE40 coding region and the single strand replication region F⁺, to generate single stranded phage DNA by cotransfection with (M13) helper phages, if desired to create derivatives of the plasmid by site directed mutagenesis (Chaudhary *et al. Proc. Natl. Acad. Sci. USA*, 87: 1066-1070 (1990)). Similarly, the plasmid pRK79K encodes the *Pseudomonas* exotoxin PE38KDEL (Chaudhary, *et al. Proc. Natl. Acad. Sci. USA*, 87: 308-312 (1990)).

The DNA sequence encoding the Fv region is inserted into the construct using techniques well known to those of skill in the art. Thus, for example, to create a plasmid for expression of the immunotoxin B3(Fv)-PE40 (pULEE3), the TGF α gene is removed and replaced by the B3(Fv) gene in a 3 fragment ligation, using an NdeI/BamHI fragment of the heavy chain coding region and the BamHI/HindIII fragment encoding the light chain Fv (Figure 1a) as described in Example 1.

Similarly, a plasmid encoding B3(Fv)-PE38 may be produced by removing the PE40 coding region from pULI1 from the HindIII site to an EcoRI site positioned just beyond the PE40 gene and replacing it with a HindIII/EcoRI fragment from pRK79K described by Chaudhary *et al. supra*. This approach is described in greater detail in Example 1.

A particularly preferred approach involves the use of plasmid pULI7 which encodes the B3(Fv)-PE38 immunotoxin (Benhar *et al. Bioconjug. Chem.*, 5: 321-326 (1994)). For each Fv, the V_H and V_L sequences are PCR amplified using the heavy chain and light chain in their respective plasmids as templates. The amplification primers are designed to have at their ends sequences that are complementary to the translation initiation, peptide linker and Fv-toxin junction (connector) which are common to the single-chain Fv-immunotoxin expression vectors. The PCR products are purified and annealed to a uracil-containing single stranded DNA corresponding to the pULI7 DNA prepared by rescue of pULI7 with a helper phage. The annealed PCR products are extended using the single stranded DNA as a template (see, for example, MUTAGENE[®] mutagenesis protocol, Biorad, Hercules, California, USA). The intact DNA may be used

to transform cells and express the new fusion protein. In a preferred embodiment, because annealing efficiency to the template is low, the remaining intact "unmodified" DNA may be digested using a restriction endonuclease which has a unique site in the B3(Fv) template but that is absent from B1 and B5. This destroys any residual B3(Fv) sequences leaving only the modified sequences. This approach is described in greater detail in Example 2.

The Preparation of DNA Encoding Variable Domain Shuffled Fusion Proteins.

It was observed that the stability of monoclonal antibody B3 could be improved. In the form of a single chain Fv immunotoxin, B3 is considerably more stable, however it still undergoes inactivation, mainly by aggregation, especially upon incubation in 0.15 M NaCl, 0.01 M NaPO₄ pH 7.4 at 37°C. In contrast to B3(Fv)-PE38 immunotoxin, B5(Fv)-PE38 is more resistant to inactivation under these conditions (see Figure 6). Based on these observations, the stability of chimeric Fv immunotoxins was examined.

It is an unexpected discovery of the present invention that chimeric Fv regions containing variable heavy and light chain domains from different, albeit related, antibodies may show significantly greater stability *in vitro* and *in vivo* than Fv regions where both the heavy and light domain are derived from the same antibody. Thus, for example, a fusion protein comprising a B3 variable heavy region and a B5 variable light region fused together and to PE38 shows higher activity and longer term stability than a B3(Fv)-PE38 fusion protein.

Nucleic acids encoding chimeric Fv regions are easily prepared using the techniques described above. The V_H and V_L sequences are PCR amplified using the heavy chain and light chain in their respective plasmids as templates as described. However, instead of using the V_H and V_L DNA from the same antibody, the V_H and V_L DNAs are selected from different antibodies. Thus, for example, one may combine a B3V_H with a B5V_L or a B5V_H with a B3V_L and so forth. The DNAs are annealed to a uracil-containing single stranded DNA corresponding to the pULI7 DNA and the synthesis of the chimeric Fv-cytotoxin fusion protein DNA is completed as described above and in Examples 2 and 12.

One of skill will appreciate that it is possible to eliminate the cytotoxin moiety and express the chimeric or single antibody Fv regions alone. These may be

used in various chemical conjugates for example, either directly with toxins or other therapeutic agents, with carriers for therapeutic agents such as liposomes, or with various labels and markers such as fluorescent labels.

5 **Stabilizing Mutations of B3**

When a more stable related form of an antibody is identified, site directed mutagenesis may be used to identify the differences between the more and less stable forms. Thus, for example the B3V_H-B5V_L-PE38 immunotoxin shows greater stability than the B3-PE38 (B3V_H-B3V_L-PE38) immunotoxin. To identify the amino acid residues contributing to the increased stability one performs a sequence analysis to identify those regions of the particular light or heavy region (in this case the V_L region) that differ from the corresponding light or heavy chain in the non-chimeric antibody. Once the differences have been identified, mutations reflecting those differences may be systematically introduced into the corresponding region of the non-chimeric antibody.

10 Comparison of the activity and stability of the mutated antibody with the chimeric antibody fusion protein will indicate which mutation is responsible for the increased stability. For example, it was discovered that replacing the B3 V_L methionine 4 with leucine stabilized the immunotoxin as much as the B3V_H-B5V_L-PE38 combination whereas replacing V_L serine 7 with threonine had no stabilizing effect. Thus, in a particularly preferred embodiment, the fusion protein comprises either B3V_H-B5V_L-PE38 or B3(Fv)-PE38: V_L M4T.

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Mutations that Increase Antibody Binding Affinity

An unexpected result of the present invention is the discovery that mutations at position 95 of the V_H region can alter the binding affinity of the single chain antibody. More specifically, it was discovered that mutations that altered the serine at position 95 in B3(Fv) to tyrosine or to phenylalanine, which are the most common amino acids at this position in other antibodies, reduced the binding affinity of B3(Fv) by approximately 10-fold (*see* Example 18). Conversely, when the tyrosine at V_H 95 in B5(fv) was mutated to serine showed a for-f ld increase binding activity as analyzed by cytotoxicity assays. B5(Fv) differed from B3(Fv) in having a completely different binding site. Thus the effect of the mutation is independent of the particular binding site.

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Without being bound to a particular theory, it is believed that a serine

located in the V_H/V_L interface slightly destabilizes the interface contacts enabling movement of V_H relative to V_L . This movement facilitates a so called "induced fit" binding mode. This destabilization mechanism would be expected to function in any antibody in which the V_H 95 position is not normally a serine. Thus, this invention
5 provides a new mechanism for increasing the binding affinity of antibodies that do not normally have a serine at V_H position 95.

Humanized B3(Fv)

Because monoclonal antibodies B1, B3 and B5 are mouse antibodies,
10 repeated administration of either labeled antibodies or the immunotoxins including these antibodies as targeting moieties will result in the formation of anti-mouse antibodies (Parren *et al.*, *Hum. Antibod. Hybridomas.*, 3: 137-145 (1992)), in addition to the production of antibodies to the toxin moiety. This immune response may preclude long term treatment in some cases. Therefore it is desirable to produce less immunogenic
15 molecules.

As a first step in making less immunogenic molecules the Fv portion of the mouse antibody is humanized so that it may then be used to replace the Fv portion of the murine antibody in the fusion proteins of the present invention. Humanized antibodies are non-human antibodies in which some or all of the amino acid residues are
20 replaced with the corresponding amino acid residue found in a similar human antibody. Humanization thereby reduces the antigenic potential of the antibody.

Antibody variable domains have been humanized by various methods, such as CDR grafting (Riechmann *et al.*, *Nature*, 332: 323-327 (1988)), replacement of exposed residues (Padlan, *Mol. Immunol.* 28: 489-498 (1991)) and variable domain
25 resurfacing (Roguska *et al.*, *Proc. Natl. Acad. Sci. USA*, 91: 969-973 (1994)), all incorporated by reference. The minimalistic approach of resurfacing is particularly suitable for antibody variable domains which require preservation of some mouse framework residues to maintain maximal antigen binding affinity. However, the straightforward CDR grafting approach has also been successfully used for the
30 humanization of several antibodies either without preserving any of the mouse framework residues (Jones *et al.* *Nature*, 321: 522-525 (1986) and Verhoeyen *et al.*, *Science*, 239: 1534-1536 (1988)) or with the preservation of just one or two mouse residues

(Riechmann *et al.*, *Nature*, 332: 323-327 (1988); Queen *et al.*, *Proc. Natl. Acad. Sci. USA*, 86: 10029-10033 (1989), all incorporated by reference.

To improve the B1, B3, or B5 antibodies or the chimeric antibodies of this invention, for therapeutic applications, the Fv portion is humanized by a method referred to as "framework exchange". In this approach, framework residues are identified that differ from human framework residues in highly homologous human V_H or V_L donors. These differing framework residues are then simultaneously mutated to human residues. The mutations are introduced onto a single-stranded DNA template prepared from a single-chain immunotoxin cassette which may be expressed in *E. coli* and allows the rapid purification and analysis of the resulting humanized variants.

This approach combines, yet deviates from the principles of CDR grafting or from the replacement of exposed residues, as some residues that are not normally exposed are humanized, while some other residues that are normally exposed are not humanized. Decisions to preserve certain mouse residues are based on knowledge regarding the effect of mutating these particular residues on the binding affinity of the Fv fragment, or on the possible interactions of these residues with other Fv residues observed in a structural model.

More specifically, humanization is accomplished by aligning the variable domains of the heavy and light chains with the best human homolog identified in sequence databases such as GENBANK or SWISS-PROT using the standard sequence comparison software as described above. Sequence analysis and comparison to a structural model based on the crystal structure of the variable domains of monoclonal antibody McPC603 (Queen *et al.*, *Proc. Natl. Acad. Sci. USA*, 86: 10029-10033 (1989) and Satow *et al.*, *J. Mol. Biol.* 190: 593-604 (1986)); Protein Data bank Entry IMCP) allows identification of the framework residues that differ between the mouse antibody and its human counterpart.

In a preferred embodiment, the mouse residues at B3 V_H positions 1, 3, 19, 24, 89 and 91 (see coordinates in Kabat *et al. supra.*) and B3 V_L positions 2, 3 and 41 (Figure 11) are preserved. In a particularly preferred embodiment, residue 82b in B3 V_H is mutated to arginine.

V_H and V_L gene segments (*e.g.* in plasmid pULI7) encoding wild type B3(Fv)-PE38 may be independently humanized by site specific mutagenesis (see Example 14). One of skill in the art will appreciate that once the Fv region has been cloned and

sequenced, alteration of various residues by site specific mutagenesis is routine using standard techniques well known to those of skill in the art (Kunkel, *Proc. Natl. Acad. Sci. USA*, 82: 488-492 (1985)).

5 **Expression of Recombinant Proteins**

The recombinant Fv regions and fusion proteins incorporating these antibody regions may be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. A particularly preferred host is *E. coli*. The recombinant protein gene will be operably linked to appropriate expression control sequences for each host. For *E. coli* this includes a promoter such as the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eukaryotic cells, the control sequences will include a promoter and preferably an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus, etc., and a polyadenylation sequence, and may include splice donor and acceptor sequences.

The plasmids of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the amp, gpt, neo and hyg genes.

Once expressed, the recombinant fusion proteins can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see, generally, R. Scopes, Protein Purification*, Springer-Verlag, N.Y. (1982), Deutscher, *Methods in Enzymology Vol. 182: Guide to Protein Purification*, Academic Press, Inc. N.Y. (1990)). Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically.

One of skill in the art would recognize that after chemical synthesis, biological expression, or purification, the single chain Fv region or a fusion protein comprising a single chain Fv region may possess a conformation substantially different

than the native protein. In this case, it may be necessary to denature and reduce the protein and then to cause the protein to re-fold into the preferred conformation. Methods of reducing and denaturing the protein and inducing re-folding are well known to those of skill in the art. (See, Debinski *et al. J. Biol. Chem.*, 268: 14065-14070 (1993); Kreitman and Pastan, *Bioconjug. Chem.*, 4: 581-585 (1993); and Buchner, *et al., Anal. Biochem.*, 205: 263-270 (1992) which are incorporated herein by reference.) Debinski *et al.*, for example, describe the denaturation and reduction of inclusion body proteins in guanidine-DTE. The protein is then refolded in a redox buffer containing oxidized glutathione and L-arginine.

One of skill would recognize that modifications can be made to the single chain Fv region and fusion proteins comprising the single chain Fv region without diminishing their biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the single chain Fv region into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids placed on either terminus to create conveniently located restriction sites or termination codons. For example, in a preferred embodiment, the primers used to construct B5(Fv) will introduce a sequence encoding an initiator methionine for expression in *E. coli* and an NdeI restriction site to facilitate cloning.

One of skill will recognize that other modifications may be made. Thus, for example, amino acid substitutions may be made that increase specificity or binding affinity of single chain Fv region and fusion proteins comprising the single chain Fv region, *etc.* Alternatively, non-essential regions of the molecule may be shortened or eliminated entirely. Thus, where there are regions of the molecule that are not themselves involved in the activity of the molecule, they may be eliminated or replaced with shorter segments that serve to maintain the correct spatial relationships between the active components of the molecule. Alternatively more flexible segments may be placed in interdomain regions which then can facilitate folding or production of the molecule (Brinkmann, *et al. Proc. Natl. Acad. Sci. USA*, 89: 3075-3079 (1992)).

Diagnostic Assays

In addition to the targeting of immunotoxins to tumors in a cancer patient, the recombinant antibodies of the present invention also recognize materials such as

surface mucins on tumor cells that would be expected to be shed into the surrounding tissues, picked up by the blood stream, and detectable in blood samples taken from distant sites. Such shed antigens have proven to be useful in the diagnosis of primary and recurrent cancers using antibodies that react to these shed antigens. A currently
5 useful example of this is the CA125 antigen that can be assayed in sera from patients with ovarian cancer to predict recurrence or to confirm a primary diagnosis of tumor. Similarly, B1, B3 and B5 may be useful in the diagnosis of tumors.

Also, the selective reactivity of these antibodies with certain types of tumor cells may be exploited for anatomic pathological diagnosis of tumors, clarifying
10 the type and origin of tumors, and whether a particular group of cells represents a recurrence of a previous tumor or the development of another primary tumor elsewhere. Such a diagnostic determination can be useful for the subsequent planning of anti-tumor therapy in each particular patient. In particular, immunohistochemical pathologic diagnosis in tissue sections (*e.g.*, biopsies) or cytological preparations (*e.g.*, Pap smears,
15 effusions) can be performed using the monoclonal antibodies of the present invention.

Another potential use of such targeting antibodies could be in the diagnosis of macroscopic foci of a tumor using antibodies B1, B3 or B5 coupled to radioisotopes that could be detected either by external body scanning (imaging diagnosis) or by localization using radiation detector probes at the time of exploratory surgery.

20 In general, the diagnostic methods described above involve contacting a B1(Fv), B3(Fv), B5(Fv) or chimeric Fv region with a biological sample either *in vivo* or *ex vivo* and subsequently detecting the binding of that antibody to the target tissue. In a preferred embodiment a diagnostic method comprises the steps of (a) removing a tissue or fluid sample from a patient; (b) adding an antibody which includes the Fv region of a
25 heavy chain of a first antibody and the Fv region of a light chain of a second antibody, where the Fv regions are recombinantly fused to form a single molecule that specifically bind a Lewis^Y-related carbohydrate antigen; and (c) detecting for the presence or absence of the antibody in the sample.

In a preferred embodiment, detection is by the detection of a label bound
30 to the antibody. Means of labeling antibodies are well known to those of skill in the art. Labels may be directly linked through a covalent bond or covalently through a linking molecule which typically bears reactive sites capable of forming covalent bonds with the label and the antibody respectively. A common approach is to label the antibody and the

label with either avidin or streptavidin r biotin which, in turn, bind irreversibly with each other.

Suitable labels are well known to those of skill in the art. The term "label", as used herein, refers to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioactive molecules such as ^{32}P , ^{14}C , ^{125}I , ^3H , and ^{35}S , fluorescent dyes such as fluorescein or rhodamine, electron-dense reagents, enzymes (as commonly used in an ELISA), luminescent enzymes such as luciferase and the like.

10 Pharmaceutical Compositions

The recombinant fusion proteins and pharmaceutical compositions of this invention are useful for parenteral, topical, oral, or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and lozenges. It is recognized that the fusion proteins and pharmaceutical compositions of this invention, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the protein with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the protein in an appropriately resistant carrier such as a liposome. Means of protecting proteins from digestion are well known in the art.

The recombinant fusion proteins and pharmaceutical compositions of this invention are particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity or lumen of an organ. The compositions for administration will commonly comprise a solution of the single chain antibody or a fusion protein comprising the single chain antibody dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the

like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of single chain antibody, fusion protein, or labeled single chain antibody in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Thus, a typical pharmaceutical composition for intravenous administration would be about 0.01 to 100 mg per patient per day. Dosages from 0.1 up to about 1000 mg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as *Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980).

The compositions containing the present fusion proteins or a cocktail thereof (*i.e.*, with other proteins) can be administered for therapeutic treatments. In therapeutic applications, compositions are administered to a patient suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health.

Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the proteins of this invention to effectively treat the patient.

Among various uses of the cytotoxic fusion proteins of the present invention are included a variety of disease conditions caused by specific human cells that may be eliminated by the toxic action of the protein. One preferred application is the treatment of cancers, in particular cancers in which the tumor cells express carbohydrate antigens that are members of the Lewis^Y family. Such cancers include, but are not limited to colon, breast, esophagus, bladder, gastric, head and neck, lung and ovarian carcinomas. The fusion proteins may also be used *in vitro*, for example, in the elimination of harmful cells from bone marrow before transplant where those cells express Lewis^Y-related antigens.

Kits

This invention also embraces kits for research or diagnostic purposes. Research kits typically include one or more containers containing the single chain antibodies of the present invention. In a preferred embodiment, research kits comprise
5 containers containing single chain B1(Fv), B3(Fv), B5(Fv), chimeric Fv, mutated Fv or humanized Fv antibodies in a form suitable for derivatizing with a second molecule, *e.g.* a label, a drug, a cytotoxin, and the like. In another embodiment, the research kits may contain DNA sequences encoding these antibodies. Preferably the DNA sequences encoding these antibodies are provided in a plasmid suitable for transfection into and
10 expression by a host cell. The plasmid may contain a promoter (often an inducible promoter) to regulate expression of the DNA in the host cell. The plasmid may also contain appropriate restriction sites to facilitate the insertion of other DNA sequences into the plasmid to produce various fusion proteins. The plasmids may also contain numerous other elements to facilitate cloning and expression of the encoded proteins.
15 Such elements are well known to those of skill in the art and include, for example, selectable markers, initiation codons, termination codons, and the like.

Diagnostic kits typically comprise containers containing the antibodies described above. The antibodies are themselves derivatized with a label or, alternatively, they may be bound with a secondary label to provide subsequent detection. As described
20 above, such labels may include radiolabels, fluorescent labels, enzymatic labels, *i.e.*, horseradish peroxidase (HRP), or the like. The kit may also contain appropriate secondary labels (*e.g.* a sheep anti- mouse-HRP, or the like). The kit may also contain various reagents to facilitate the binding of the antibodies, the removal of non-specific binding antibodies, and the detection of the bound labels. Such reagents are well known
25 to those of skill in the art.

Methods for using the research and diagnostic kits described above are generally well known, and will generally be provided in an instruction manual for use of the kit.

EXAMPLES

The following examples are offered by way of illustration, not by way of limitation.

Example 1

Cloning of DNA Fragments Encoding the Heavy and Light Fv Region of MAb B3

B3 cloning experiments and propagation of plasmids were carried out initially in *E. coli* HB101 (Boyer *et al.*, *J. Molec. Biol.* 41: 459-72 (1969)). DNA fragments encoding the Fv portions of heavy and light chain of monoclonal antibody (MAb) B3 were obtained by (PCR[®]) amplification of single stranded DNA which was synthesized by random primed reverse transcription of mRNA from a B3 monoclonal antibody producing hybridoma cell line. Polymerase chain reaction (Saiki *et al.*, *Science*, 239: 487-91 (1988)) was performed using the Perkin Elmer GeneAmp kit and an Perkin Elmer/Cetus thermocycler, under conditions as described in Chaudhary *et al.*, *Proc. Natl. Acad. Sci. USA* 87: 1066-70 (1990).

The primer pair B3-H1 and B3-H2 was used for amplification of the heavy chain Fv coding region, while the primer pair B3-L1 and B3-L2 was used for amplification of the light chain Fv coding region (see Table 1 and Sequence ID Nos. 1, 2, 7, and 9). These oligonucleotides have at their 3' ends constant sequences that occur at the beginning and end of mouse Fv DNA. At their 5' ends are restriction endonuclease recognition sites (NdeI, BamHI, HindIII) for cloning of the PCR products as shown in Fig. 1a. The products of the amplifications of heavy- and light chain Fv DNA fragments were identified by agarose gel electrophoresis to be DNA fragments between 350 and 400 bp. They were purified from gels, cut with BamHI or HindIII (Fig. 1a) and, after purification on a second gel, ligated with HindIII- or BamHI linearized and dephosphorylated pBR322 vector (Bolivar *et al.*, *Gene*, 2: 95-113 (1977)). The nucleotide sequence of the light- and heavy chain Fv coding region of monoclonal antibody B3 was determined from double stranded plasmid DNA using sequencing primers (New England Biolabs, Beverly, Massachusetts, USA) adjacent to the BamHI or HindIII site of pBR322 and a T7 polymerase sequencing reagent kit (United States Biochemicals, Cleveland, Ohio, USA).

Tabl 1. PCR primers used to amplify Fv heavy and light chains. Th Frl primers were designed according t the amino acid sequences which were determined by Edman degradation, and are indicated in single letter code abov the primer sequences. Underlined Met ar initiator methionine cod ns. Other underlined amin acids in the light chain primers are segments of the peptide linker that fuses V_H t V_L in the single chain configuration. Underlined nucleotides in B1HFr1 and B5HFr1 encode the initiator methionine for expression in *E. coli*, and include an *NdeI* restriction site. Underlined nucleotides in B1LFr4 and B5LFr4 are complementary to the coding sequence for segments of the peptide linker that fuses V_H to V_L in the single-chain configuration. Underlined nucleotides in B1LFr4 B5LFr4 are complementary to the coding sequence of the junction between the Fv and PE38 and include a *HindIII* restriction site.

Sequence		
Seq. Name	5'-3'	ID
<u>Heavy chain primers</u>		
B3-H1	TAACTAGGATCCGTCCATATGGATGTGAAGCTGGTGGAG-TCTGG	1
B3-H2	TGGATAGACTGATGGGGATCCGCCTCCGCCTGAGGAGAC	2
B1HFr1	<div>M E V Q L V E S G G</div> GATATACATATGGAGGTGCAGCTGGTGGAAATCTGGAGGA	40 3
B5HFr1	<div>M E V K L V E S G G</div> GATATACATATGGAGGTGAAGCTGGTGGAAATCTGGAGGA	41 4
GammaCH1	AGCAGATCCAGGGGCCAGTGGATA	5
B1HFr4	<u>ACCGGATCCGCCT</u> GCAGAGACAGTGAC	6
B5HFr4	<u>ACCGGATCCGCCT</u> CCGCCTGAGGAGACAGTGAC/G	7
<u>Light chain primers</u>		
B3-L1	GTCTCCAAGCTTGGGGATCCGGTGGTGGCGGATCTGGAGG-TGGCGGAAGCGATGTGCTGACCCAGTCTCC	8
B3-L2	AGTTGGTGCAGCATCAAAAGCTTT[G/T]A[G/T][T/C]-TCCAGCTT[T/G]GT[G/C]CC	9
B3-L3	TTGGGGATCCGGTGGTGGCGGATCTGGA	10
B3-L4	AGCGGGAATTCATTATTTAATTTCCAGCTTTGTCCCCGAC	11
B1LFr1	<div>G G G S D V V M T Q</div> <u>GGTGGCGGAAGCGATGTTGTGATGACCCAA</u>	42 12
B5LFr1	<div>G G G S D V L L T Q</div> <u>GGTGGCGGAAGCGATGTTTGTGATGACCCAA</u>	43 13
C-kappa	TGGTGGGAAGATGGATACAGTTGG	14
B1LFr4	<u>GGAAGCTTT</u> CAGCTCCAGCTTGGT	15
B5LFr4	<u>GGAAGCTTT</u> ATTTCCAACTTTGT	16

Example 2**Cloning of DNA Fragments Encoding the Heavy and Light Fv Segments of MAbs B1 and B5**

To obtain DNA encoding the variable regions of the heavy and light chains of B1 and B5, Poly(A)⁺ mRNA was prepared from 10⁵ hybridoma cells and reverse-transcribed using random hexamers as primers to yield first strand cDNA. Separate PCR[®] reactions were carried out to amplify fragments encompassing heavy chain variable through part of CH1 domains, and light chain variable through part of C-kappa. The B1 and B5 V_H sequences were amplified using 5' end primers designed according to the amino-terminal protein sequence of the B1 and B5 heavy chains and 3' end primers designed according to consensus immunoglobulin constant region sequences (Kabat *et al.* (1991) *supra.*). In particular, B1 V_H was amplified using 5' end primer B1HFr1 and 3' end primer GammaCH1 (Table 1, Sequence ID Nos. 3 and 5 respectively). B5 V_H was amplified using 5' end primer B5HFr1 and 3' end primer B5HFr4 (Table 1, Sequence ID Nos. 4 and 7 respectively). Primer GammaCH1 was designed according to consensus IgG1 CH1 region codons 122-129 while primer B1HFr4 was designed according to the determined nucleotide sequence of codons 109-113 of B1 V_H (Kabat *et al.*, (1991) *supra.*).

The B1 and B5 V_L sequences were amplified using 5' end primers B1LFr1 and B5LFr1 (Table 1 and Sequence ID Nos. 12 and 13) which were designed according to the amino-terminal protein sequence of B1 and B5 light chains respectively (Sequence ID Nos. 20 and 22) in combination with the primer C-kappa (Table 1, Sequence ID No. 14). Primer C-kappa was designed according to consensus kappa light chain codons 113-120 (*Id.*).

PCR was performed as described by Brinkmann *et al.*, *Proc. Nat. Acad. Sci. USA* 88: 8616-8620 (1991).

The crude PCR products were subcloned into a PCR[®] cloning vector (Invitrogen, San Diego, California, USA) employing blue/white selection. Clones containing the correct size insert by DNA restriction analysis were identified. The nucleotide sequence of the heavy or light chain coding regions was determined from double stranded plasmid DNA using sequencing primers (Invitrogen) adjacent to the PCR EcoRI cloning site and the Sequenase[™] kit (United States Biochemical Corp). Three to five independent clones were sequenced for each amplified DNA segment. The

nucleotide sequences of the B1 V_H and V_L are shown in Sequence ID Nos. 19 and 20 respectively, while the nucleotide sequences of the B5 V_H and V_L are shown in Sequence ID Nos. 21 and 22 respectively. B5 could not be amplified using a mu chain CH1 primer, so the consensus heavy chain B5HFr4 primer (Table 1, Sequence ID No. 7) was used instead. V_H Primer B5HFr4 was designed according to consensus IgG1 Fr4 region codons 109-113 (Kabat *et al.*, (1991) *supra*.)

Alignment of B1, B3 (Brinkmann *et al.*, *Proc. Nat. Acad. Sci. USA* 88: 8616-8620 (1991)), and B5 Fv sequences revealed that B5 is highly homologous to B3 (91.6% identity in V_H and 94.9% identity in V_L coding sequence) and to the anti-Lewis^x antibody H18A (Kaneko *et al.*, *J. Biochem.*, 113: 114-117 (1993)) (93.3% identity in V_H and 97.6% identity in V_L coding sequence). B1 differs considerably both in framework and in CDR sequence from both B3 (83.9% identity in V_H and 88.9% identity in V_L coding sequence) and B5 (86.3% identity in V_H and 91.2% identity in V_L coding sequence), and does not show high sequence identity to any known anti-carbohydrate antibody in a database search (Devereux *et al.*, *Nucleic Acids Res.*, 12: 387-395 (1984)). All three antibodies have a mouse class III heavy chain and a kappa II light chain (Kabat *et al.*, *supra*). The differences in sequence between B1 and B3 may explain why they recognize different epitopes of otherwise similar antigens (*see*, Pastan *et al.*, *Cancer Res.*, 51: 3781-3787 (1991) and U.S. Patent No. 5,242,813).

Example 3

Construction of Plasmids for Expression of B3(Fv) and B3(Fv)-Immunotoxins

A) Construction of B3(Fv) and B3(Fv)-PE40

The expression plasmid pVC38H contains the gene from the immunotoxin TGF α -PE40 under control of the T7 promoter (Chaudhary *et al.*, *Proc. Natl. Acad. Sci. USA* 87: 1066-70 (1990)), the Tc transcription terminator at the 3' end of the PE40 coding region and the single strand replication origin, F⁺, to generate single stranded phage DNA by cotransfection with (M13) helper phages, if desired, to create derivatives of the plasmid by site directed mutagenesis. The TGF α coding region in pVC38H has an NdeI recognition site at the 5' end and a HindIII site at the point of connection to the DNA encoding PE40.

To create a plasmid for expression of the immunotoxin B3 (Fv)-PE40 (pULEE3), the TGF α gene was removed and replaced by the B3(Fv) gene in a 3 -

fragment ligation, using an NdeI/BamHI fragment of the heavy chain coding region and the BamHI/HindIII fragment encoding the light chain Fv (Fig. 1a). Because sequence analysis showed a mutation (deletion and frameshift) at the 5' end of the light chain Fv gene due to a sequence repetition in the PCR primer annealing region, site-directed mutagenesis was performed (Kunkel, *Proc. Natl. Acad. Sci. USA*, 82: 488-92 (1985)),
5 using uridine incorporated single stranded phagemid DNA (pULEE3) as the mutagenesis template. In the resulting plasmid (pULI1), the correct amino end of the B3 light chain established by partial protein sequencing of monoclonal antibody B3, was reconstructed.

To make another B3(Fv) immunotoxin, B3(Fv)-PE38DKEL, the PE40
10 coding region was removed from pULI1 from the HindIII site to an EcoRI site positioned just beyond the PE40 gene, and replaced by a HindIII/EcoRI fragment from pRK79K encoding the PE variant PE38KDEL which lacks domain Ia (amino acids 1-252) and part of domain Ib (amino acids 365-380), and also contains an altered carboxyl terminal sequence KDEL (Chaudhary *et al.*, *Proc. Natl. Acad. Sci.*, 87: 308-12 (1990)). The
15 expression plasmid pULI4 for production of B3(Fv) was constructed by removal of the light chain and PE40 coding region from pULI1 from BamHI to EcoRI which was replaced by a PCR fragment obtained by amplification of the light chain Fv coding sequence with the primer-pair B3-L3 + B3-L4. The primer B3-L3 (Table 1) is similar to B3L1, used for cloning of light chain Fv from cDNA and B3-L4 (Table 1) is, in the 3'
20 part for priming the PCR, identical to B3-L2, but, at the 5' end, the HindIII site for fusion to PE-sequences is replaced by translation stop codons followed by an EcoRI recognition sequence.

B) Construction of pULI7, the Plasmid for Expression of LMB7. (B3(Fv)-PE38 with C3-Connector
25

B3(Fv)-PE38, also called LMB7 is one recombinant B3(Fv)-immunotoxin of this invention preferred for use as a cancer therapeutic. The plasmid pULI7 for expression of B3(Fv)PE38 was constructed as follows: Plasmid pULI1 contains the Fv region of monoclonal antibody B3 in the form of a single chain Fv containing a
30 (Gly₄-Ser)₃ peptide linker between V_H and V_L, fused to PE-40, a truncated form of *Pseudomonas* exotoxin (see Brinkmann *et al.*, *Proc. Nat. Acad. Sci. USA* 88: 8616-8620 (1991) and Fig. 1b). To improve folding and producti n of this molecule, a flexible "connector" peptide, designated C3, was added between the Fv and th toxin moiety by

PCR mutagenesis (Brinkmann, *et al. Proc. Natl. Acad. Sci. USA* 89: 3075-3079 (1992)) to result in pULI6. Finally, part of the toxin portion of pULI6 was replaced with a shorter molecule with the same activity, PE38 (lacking domain Ib of PE), replacing by subcloning a SalI-EcoRI toxin fragment of pULI6 with the PE38 coding SalI-EcoRI fragment of pCS10 (Siegall *et al. J. Biol. Chem.*, 264: 14256-14261 (1989)). The resulting expression plasmid, which codes for the immunotoxin B3(Fv)-PE38 is pULI7 (Fig. 1b).

Example 4

Construction of Plasmids for Expression of B1(Fv)- and B5(Fv)-Immunotoxins

For expression as single-chain immunotoxins, B1 and B5 Fv fragments replaced B3Fv sequences in the expression plasmid pULI7 which encodes the B3(Fv)-PE38 immunotoxin (Benhar *et al. Bioconjug. Chem.*, 5:321-326 (1994)). For each Fv, the V_H sequences were PCR amplified using the heavy chain clones in PCR[®] plasmids as templates. Primers B1HFr1 and 5'-phosphorylated B1HFr4 were used to amplify B1V_H, while primers B5HFr1 and 5'-phosphorylated B5HFr4 were used to amplify B5V_H. The V_L sequences were amplified using the light chain clones in PCR[®] plasmids as templates. Primers B1LFr1 and 5'-phosphorylated B1LFr4 were used to amplify B1V_L, while primers B5LFr1 and 5'-phosphorylated B5LFr4 were used to amplify B5V_L. The primers had at their ends sequences that are complementary to the translation initiation, peptide linker and Fv-toxin junction (connector) which are common to the single-chain Fv-immunotoxin expression vectors. Primers B1LFr4 and B5LFr4 were designed according to the determined nucleotide sequence of codons 102-107 of B1 and B5 V_L respectively. The PCR amplifications were performed as described in Example 2.

The PCR products were purified using spin columns, combined and annealed to a uracil-containing single-stranded DNA phagemid pULI7 which encodes the single-chain immunotoxin B3(Fv)-PE38. The phagemid was prepared by rescue of pULI7 phagemid with an M13MK07 helper phage (Bio-Rad, Hercules, California, USA). The DNA was extended and ligated according to the MUTA-GENE[®] mutagenesis kit protocol (Bio-Rad).

Since the annealing efficiency of the PCR fragments to the single-stranded template, and hence the mutagenesis efficiency was relatively low (about 10%), an

additional step was added. Plasmid DNA obtained from a pool of transformants from the mutagenesis reaction was digested with a restriction endonuclease which had a unique site in the B3Fv template, but whose site was absent from both B1 and B5. The digested DNA was used to re-transform *E. coli* cells. Following this extra step mutants were
5 obtained with an efficiency greater than 80%. Correct clones were identified by DNA restriction analysis and verified by DNA sequencing. The resulting immunotoxin clones were named pB1(Fv)-PE38 and pB5(Fv)-PE38.

Example 5

10 Expression and Purification of Recombinant B3(Fv)-Immunotoxins

Plasmids were transformed in the expression-host *E. coli* BL21 (λ DE3) (Studier *et al. J. Mol. Biol.* 189: 113-30 (1986)). The bacteria were grown in superbrotch containing 0.2-0.4% glucose, 0.05 % MgSO_4 , and 100 $\mu\text{g/ml}$ ampicillin, induced in the log phase at OD_{600} of 3.0 with 1 mM isopropyl-B-D-thiogalactopyranoside
15 (IPTG) and harvested 90 min later. About 30% of the total protein of the induced cultures was the recombinant expression product which was deposited in inclusion bodies. The purified inclusion bodies contained almost pure recombinant protein, which had the expected size of about 67 kDa for a single chain immunotoxin. The recombinant immunotoxin molecules were solubilized, refolded, purified, and the protein was
20 analyzed as previously described (Chaudhary *et al., Nature* 339: 394-97 (1989) & Batra *et al., J. Biol. Chem.* 265: 15198-202 (1990)). Protein concentrations were determined by Bradford assay (Bradford, *Anal. Biochem.* 72: 848-54 (1976)).

Example 6

25 Expression and Purification of B1(Fv)-PE38 and B5(Fv)-PE38

Cultures of *E. coli* (BL21 λ DE3, see Studier, *et al. J. Mol. Biol.* 189: 113-130 (1986)) were transformed with each expression plasmid to produce B1(Fv)- and B5(Fv)- immunotoxins. Following IPTG induction, the overproduced fusion proteins accumulated in inclusion bodies. These were isolated by solubilization and refolding of
30 inclusion body protein using redox-shuffle as described (Buchner *et al., Anal. Biochem.* 205, 267-270 (1992)). Briefly, inclusion bodies were dissolved in 6 M guanidine (HCl)/0.1 M Tris(HCl) Ph 8.0/2 mM EDTA and reduced by the addition of solid DTE to a final concentration of 65 Mm at a protein concentration of 10 mg/ml. The

solubilized and reduced inclusion body proteins were diluted x 100 into 0.1 M Tris (HCl) Ph 8.0/0.5 M L-arginine/0.9 M oxidized glutathione/2 M EDTA and were allowed to refold for 36 hr at 10°C. The refolded proteins were extensively dialyzed against 20 M Tris (HCl) Ph 7.4/2 M EDTA/0.1 M Urea. Properly refolded proteins were separated from contaminating proteins and aggregates by sequential ion-exchange chromatography on Q-sepharose and Mono Q columns (Pharmacia, Piscataway, New Jersey, USA) followed by size exclusion chromatography on a TSK G3000SW (TosoHaas, Montgomeryville, Pennsylvania, USA) column. Typically, purified monomeric proteins were over 95% pure.

Example 7

Cytotoxic Activity of Chemically Linked and Recombinant B3(Fv)-Immunotoxins

Assays measuring inhibition of protein synthesis were performed as previously described (Chaudhary *et al.*, *Nature*, 339: 394-97 (1989) and Batra *et al.*, *J. Biol. Chem.* 265: 15198-202 (1990)). All assays were performed in 96 well plates each well containing 1.6×10^4 cells in 200 μ l medium. For competition assays designed to prove the specificity of the recombinant immunotoxins, the medium was changed and 50 μ g/well of antibody was added 15 min prior to the addition of the immunotoxin.

As shown in Figure 3 and in Table 1, the recombinant single chain immunotoxins inhibited protein synthesis in cells expressing the B3 antigen but not in non-expressing cells, similarly to the previously described results with chemical conjugate of B3 with a truncated form of PE (Pai *et al.*, *Proc. Natl. Acad. Sci. USA*, 88: 3358-62 (1991)). The relative potencies of the chemical conjugate and the single chain immunotoxins were about the same on the four antigen positive cell lines MCF7, CRL1739, A431 and LNCaP. The most active agent was B3(Fv)-PE38KDEL.

Tabl 1. Activities of B3 immunotoxins on different cell lines.
Cytotoxicity (ID₅₀) in ng/ml (pM).

Cell Lin	Cancer Type	B3 antigen	B3(Fv)-PE40KDEL	B3(Fv)-PE38	B3-LysPE40
MCF7	breast	++	3 (50)	0.2 (3.2)	3 (16)
CRL1739	gastric	++	3 (50)	0.3 (5)	3 (16)
A431	epidermoid vulva	+	3 (50)	0.8 (13)	8 (42)
LNCaP	prostate	+	40 (1330)	20 (325)	85 (460)
KB3-1	epidermoid cervix	-	>1000	>1000	>1000
HUT102	adult T cell leukemia	-	>1000	>1000	>1000

The recombinant single chain B3-Fv immunotoxins did not affect B3 antigen-negative control cells. The cytotoxicity of the recombinant B3(Fv)-PE40 (ID₅₀ = 50 pM; 3.0 ng/ml) was similar to the chemically linked B3-immunoconjugate (ID₅₀ = 42 pM; 8 ng/ml), whereas B3(Fv)-PE38KDEL was much more active than the chemical conjugate (ID₅₀ = 13 pM; 0.8 ng/ml). This is despite the fact that the single chain immunotoxins possess only one antigen binding site per molecule and the chemical conjugate has two (see Table 2 below).

Table 2. Structure and Activity of B3 Immunotoxins on A431 Cells.

Immunotoxin	Toxin Part	C-Term	Binding	ID ₅₀
B3 chemical conjugate	PE40	REDLK	bivalent	8.0 ng/ml (42 pM)
B3(Fv) fusion protein	PE40	REDLK	monovalent	3.0 ng/ml (50 pM)
B3(Fv) fusion protein	PE38	KDEL	monovalent	0.8 ng/ml (13 pM)

B3(Fv)-PE38KDEL has two features that distinguish it from B3(Fv)-PE40. One is that a portion of domain Ib encompassing amino acids 365-380 is deleted. This removes the disulfide bond formed between cysteine residues at positions 372 and 379, which might form disulfide bonds with other cysteines during the renaturation process and thereby result in the creation of inactive chimeric toxins. The second feature is that

the carboxyl terminus of the toxin is changed from the original sequence REDLK to KDEL. When the disulfide bond was removed in their molecules, the increase in activity was small. For example, TGF α -PE38 is only 50% more active than TGF α -PE40 (see Siegall *et al.*, *J. of Biol. Chem.* 264: 14256-14261 (1989)). IL6PE38 is no more active than IL6-PE40. Changing REDLK to KDEL usually only produces a two to three fold increase in activity of chimeric toxins.

To analyze whether the cytotoxicity of B3(Fv)-immunotoxins was specific, competition experiments were carried out with an excess of monoclonal antibody B3. The data in Figure 3(b) shows that the intoxication of A431 carcinoma cells by B3(Fv)-PE38KDEL is due to the specific binding to the B3 antigen, since its cytotoxicity was blocked by excess B3 but not by MAb HB21 which recognizes the transferrin receptor on these cells (Haynes *et al.*, *J. Immunol.*, 127: 347-51 (1981)). A large excess of monoclonal antibody B3 is necessary for reversal of cytotoxicity, probably because there is a large amount of the B3 antigen on the surface of A431 cells (Pai *et al.*, *supra.*)

Example 8

Antigen Binding, ADP-Ribosylation and Specific Cytotoxicity of Recombinant Immunotoxins

A) ADP-Ribosylation Activity

The ADP-ribosylation activity of each of the immunotoxins was tested to verify that they were of equal enzymatic activity. ADP-ribosylation activity was determined by the incorporation of [¹⁴C]-NAD into acid-precipitable material using elongation factor 2 enriched wheat-germ extract (Collier and Kandel, *J. Biol. Chem.*, 246: 1496-1503 (1971)). As shown in Fig. 4(A), B3(Fv)-PE38, which was used as a reference molecule, B1(Fv)-PE38 and B5(Fv)-PE38 had similar ADP-ribosylation activities.

B) Specific Cytotoxicity

Cytotoxicity towards A431 cells was measured by the inhibition of incorporation of [³H]-leucine into cell protein, following 2 hours or 20 hours of incubation of the cells with serial dilutions of immunotoxins in PBS + 0.2% BSA (see Brinkmann *et al.*, *Proc. Natl. Acad. Sci. USA*, 88: 8616-8620 (1991)). As shown in Fig. 4(B), when tested on A431 cells which strongly bind the B3 and the B1 MAbs,

B3(Fv)-PE38 has an IC₅₀ of 2.8 ng/ml and 2.0 ng/ml following 2 or 20 hours incubation respectively. B1(Fv)-PE38 has an IC₅₀ of 0.6 ng/ml and 0.3 ng/ml following 2 or 20 hours incubation respectively. B5(Fv)-PE38 has an IC-50 of 120 ng/ml and 20 ng/ml following 2 or 20 hours incubation respectively.

To check the specificity of the immunotoxins, the same cytotoxic assay (Brinkmann *et al. supra.*) was done on additional cell lines. As shown in Table 3, B3(Fv)-PE38, B1(Fv)-PE38, and B5(Fv)-PE38 had the same spectrum of recognition of the cancer cell lines tested albeit having different levels of cytotoxic activity toward the antigen-positive cells, which correlates with the binding affinity of each immunotoxin toward its cellular binding site. These cell lines differ in their level of B3 or B1 antigen expression (Pastan *et al.*, 1991; Brinkmann *et al.*, 1993; see Table 3).

Table 3. Cytotoxicity of B1(Fv)PE38, B3(Fv)PE38, and B5(Fv)PE38 toward various cell lines.

Cell Line	Source	B1 or B3 antigen expression	B1(Fv)-PE38	B3(Fv)-PE38	B5(Fv)-PE38
A431	Epidermoid carcinoma	+++	0.3	2.0	20
MCF7	Breast carcinoma	+++	0.6	4.0	22
LnCap	Prostate carcinoma	+	2.7	21	210
KB 3-1	Cervical carcinoma	-	>1000	>1000	>1000
HUT102	T-cell leukemia	-	>1000	>1000	>1000
L929	Mouse fibroblast	-	>1000	>1000	>1000

C) Antigen Binding Affinity of B1(Fv)-PE38 and B5(Fv)-PE38

The specific antigen binding of the immunotoxins was further analyzed by determination of their binding affinity to antigen positive cells by competition assays, in which increasing concentrations of each immunotoxin were used to compete the binding of iodinated B3 IgG or B1 IgG to A431 adenocarcinoma cells at 4°C as described by Benhar *et al.*, *Bioconjug. Chem.*, (1994) *supra.* As shown in Figure 5, B1(Fv)-PE38 competed for the binding of [¹²⁵I]-B1 IgG to A431 cells by 50% at 1.3 μM, and for the binding of [¹²⁵I]-B3 IgG by 50% at 1.7 μM. B3(Fv)-PE38 competed for the binding of

[¹²⁵I]-B1 IgG to A431 cells by 50% at 2.7 μM, and for the binding of [¹²⁵I]-B3 IgG by 50% at 2.5 μM. B5(Fv)-PE38 competed for the binding of [¹²⁵I]-B1 IgG to A431 cells by 50% at about 50-100 μM, and for the binding of [¹²⁵I]-B3 IgG by 50% at 50 μM. B1 IgG competed by 50% for the binding of ¹²⁵I labeled B1 IgG at 110 nM and B3 IgG competed by 50% for the binding of ¹²⁵I labeled B3 IgG at 200 nM (not shown).

The analyses of the B1(Fv)-PE38 and B5(Fv)-PE38 and their comparison with B3(Fv)-PE38 showed that all three had similar ADP-ribosylation activities (Fig 4(A)), indicating that cytotoxic activity differences between the immunotoxins did not result from different enzymatic activity, but instead reflect relative antigen binding affinities. The cytotoxic assays (Fig. 4(B) and Table 3), show that the cytotoxic activity of B1(Fv)-PE38, B3(Fv)-PE38, and B5(Fv)-PE38, is specific, as they all kill antigen positive cells, whose sensitivity to intoxication is proportional to the level of antigen expression, while antigen-negative cells are spared. The activities of the immunotoxins varied with B1(Fv)-PE38 being the most potent. In a 20 hr assay B1(Fv)-PE38 had an IC₅₀ of 0.3 ng/ml on A431 cells, and B5(Fv)-PE38 was the least potent, with an IC₅₀ of 20 ng/ml on A431 cells.

The antigen binding assays (Fig. 5) showed that apparently B1 and B5 recognize the same antigen as B3, because all three immunotoxins compete for the binding of ¹²⁵I labeled B1 IgG and B3 IgG. However, the possibility of each recognizing a different epitope of a mutual antigen can not be excluded. A clear correlation was observed between each immunotoxins' antigen binding affinity and its cytotoxic potency. The relative low affinity of B5(Fv)-PE38 is consistent with its being derived from an IgM.

Example 9

Stability of Immunotoxins

The stability of the B1(Fv)-, B3(Fv)- and B5(Fv) immunotoxins following heat treatment was determined by incubation at 0.1 mg/ml in PBS at 37°C for 4 hours, followed by analytical chromatography on a TSK G3000SW (TosoHaas) column, to separate the monomers from the aggregates. Cytotoxic activities of aliquots of heat treated immunotoxins were determined as described above, and compared to the activities of the untreated immunotoxins.

As shown in Fig. 6(A), all three immunotoxins were monomeric before the incubation (Fig. 6(A), broken lines), whereas after 4 hours of incubation in PBS at

37°C, about half B1(Fv)-PE38 and B5(Fv)-PE38 were aggregated, and B3(Fv)-PE38 was completely aggregated (Fig. 6(A), solid lines). As shown in Fig. 6(B), following the 4 hours 37°C treatment, B1(Fv)PE38 had an IC₅₀ of 1.8 ng/ml which is 25% of its cytotoxic activity before treatment. B5(Fv)-PE38 had an IC₅₀ of 30 ng/ml (Fig. 6(B)) which is 66% of its cytotoxic activity before treatment. No cytotoxic activity could be detected after treatment of B3(Fv)-PE38 for 4 hours at 37°C.

These stability assays reveal differences in stability among the single-chain Fv-immunotoxins tested here. This is evident both from the cytotoxicity assay (Fig. 6(B)) and the from the stability assay (Fig (6)). The B3(Fv)-PE38 is somewhat unstable at 37°C (Benhar *et al.*, 1994 *supra*; Brinkmann *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 7538-7542 (1993)) and undergoes inactivation mainly by aggregation. As a consequence it shows little difference in cytotoxic activity when incubated 2 or 20 hours on A431 cells, because most of the immunotoxin is inactivated after 2 hours. B1(Fv)-PE38 is more stable, as indicated by the fact that its cytotoxic activity following 20 hours incubation on A431 cells is twice the activity following a 2 hour incubation, and by its reduced aggregation and inactivation following incubation at 37°C. B5(Fv)-PE38 may be the most stable as its cytotoxic activity following 20 hours incubation on A431 cells is six fold higher than the activity after a 2 hour incubation. B5(Fv)-PE38 seems to aggregate as much as B1(Fv)-PE38 in the absence of antigen, but, when incubated with cells, it appears to be more resistant than both B1(Fv)-PE38 and B3(Fv)-PE38 to inactivation following incubation at 37°C. Since the antigen binding studies were done at 4°C for three hours, conditions under which all three immunotoxins are stable, the relative binding affinities of the immunotoxins best correlate with their relative cytotoxic activities following a 2 hour incubation period.

Example 10

Assay of Blood Levels of B3(Fv)-PE38KDEL in Mice

Six week old (19-20 gm) female Balb/c mice were injected with 10 µg of B3(Fv)-PE38KDEL in the tail vein. Blood was drawn at various time intervals and the level of the immunotoxin measured by incubating serum with A431 cells and measuring inhibition of protein synthesis. A standard curve was made with pure B3(Fv)-PE38KDEL and the blood level of immunotoxin (which is shown in Figure 7) calculated using this curve.

Example 11

Anti-tumor Activity of B3(Fv)-PE38KDEL in Nude Mice Bearing a Human Epidermoid Carcinoma

A431 cells (3×10^6) were injected subcutaneously on day 0 into female nude mice (4-6 weeks old, 18-20 gm). Mice with 5 mm by 5 mm tumors, that usually developed by day 4, were treated with B3(Fv)-PE38KDEL or, as a control, with MAbB3 or antiTac(Fv)-PE38KDEL (Chaudhary *et al.*, *Nature* 339: 39497 (1989)). Because the lifetime of B3(Fv)-PE38KDEL in the circulation of the mice was observed to be only 15-20 min (Figure 7), six injections were given at 12 hour intervals into the tail vein, starting 4 days after tumor implantation. Each treatment group consisted of five animals. The volume of the tumor was calculated by (tumor volume in $\text{cm}^3 = \text{length} \times \text{width}^2 \times 0.4$).

As shown in Figure 8, injection of either 2.5, 5 or 10 μg twice daily produced complete tumor regression despite the fact that B3(Fv)-PE38KDEL has a short lifetime (15-20 min) in the circulation. Partial regression was observed when only 0.5 μg was injected. No toxicity was observed at these doses. In addition, when mice with large tumors about 1 cm in diameter were treated with 5 μg twice a day for 4 days, complete regression of these large tumors containing about 5×10^6 cells rapidly occurred (Figure 8(D)). Previously, it was found that even the administration of 75 μg per day for 5 days of a chemical conjugate composed of B3 and PE40 (see Table 2) only produced partial regression of large tumors despite the fact that the chemical conjugate has a much longer lifetime in the blood (4 hours). The recombinant molecule probably has a higher antitumor activity in the mouse model because of its small size which allows better access to tumor cells. Regression of MCF-7 tumors (breast carcinoma) also was observed with 5 μg twice daily of B3(Fv)-PE38KDEL.

Example 12

Chimeric Fv Region Immunotoxins and Mutated Immunotoxins Show Increased Stability

In order to investigate the mechanism contributing to the greater stability of B5(Fv)-PE38 as compared to B3(Fv)-PE38 immunotoxins comprising chimeric Fv regions in which the light and heavy chains were derived from different antibodies were constructed as described below. In addition, B3(Fv)-PE38 immunotoxins carrying V_L

mutations M4L (in which methionine 4 is replaced with leucine) and S7T (in which serine 7 is replaced with threonine) in combination or separately were prepared. The cytotoxicity and stability of the chimeric Fv and mutated fusion proteins was then determined.

5

A) Cloning and Expression of Chimeric Fv and Mutated Immunotoxins

To produce chimeric Fv immunotoxins, DNA encoding the variable regions of the heavy and light chains of B5 was prepared from mRNA obtained from B5 hybridoma cells as described in Example 2. To generate single chain immunotoxins with
10 Fvs of B5, the V_H and V_L fragments were PCR amplified using phosphorylated primers to enable the ligation of extended PCR products (see Example 2). The resulting PCR products were used as "primers" in a "domain shuffling" scheme where they replaced the corresponding B3(Fv) V_H or V_L regions or both, generating single chain Fv-toxin expression plasmids having B3 V_H -B5 V_L , B5 V_H -B3 V_L , and B5Fv (Figure 9). The
15 extension of template-primer, ligation, transformation and analysis of clones are described in Examples 2 and 4. This procedure resulted in the generation of plasmids for expression in *E. coli* in which either the V_H or the V_L domains of B3 were replaced by the corresponding domains from B5 (Figure 9).

In addition, plasmids expressing B3(Fv)-PE38 derivatives in carrying V_L
20 mutations M4L and S7T in combination or separately, were prepared by site-specific mutagenesis (Kunkel, *et al. Proc. Natl. Acad. Sci. USA*, 82: 488-492 (1985)). In addition, similar plasmids expressing B3(Fv)-PE38 derivatives carrying V_L mutations M4L and S7r together or separately were prepared by site-specific mutagenesis using oligonucleotide primers.

25 Plasmids encoding B3(Fv)-PE38, B5 V_H -B5 V_L -PE38, B5 V_H -B3 V_L -PE38, B5(Fv)-PE38 or mutated B3(Fv)-PE38 were expressed and the fusion protein purified as described in Example 6. Typically, monomeric proteins were recovered that were over 95% pure.

B) Specific cytotoxicity of recombinant immunotoxins.

The cytotoxic activity of B3(Fv)-PE38 and its derivatives was assessed, according to the method of Brinkmann *et al.*, *Proc. Nat. Acad. Sci. USA* 88: 8616-8620 (1991), by measuring the incorporation of [3 H]-leucine by various human carcinoma cell

lines after treatment with serial dilutions of the immunotoxin in phosphate buffered saline (PBS) containing 0.2% BSA as described in Example 8.

When tested on A431 cells, which strongly bind monoclonal antibody B3, B3(Fv)-PE38 has an IC_{50} of 2.8 ng/ml and 2.0 ng/ml following 2 or 20 hours incubation respectively. B3V_H-B5V_L-PE38 and B3(Fv)-PE38 V_L: M4L S7T are more active and have identical IC_{50} s of 0.6 ng/ml and 0.3 ng/ml following 2 or 20 hours incubation respectively. B5(Fv)-PE38 is much less active with an IC_{50} of 120 ng/ml and 20 ng/ml following 2 or 20 hours incubation respectively. B5V_H-B3V_L-PE38 has an IC_{50} of 200 ng/ml and 120 ng/ml following 2 or 20 hours incubation respectively (data not shown).

C) Stability of the recombinant immunotoxins.

The stability of B3V_H-B5V_L-PE38, and B3(Fv)PE38;V_L M4L S7T were tested and compared to that of B3(Fv)-PE38 by determination of their respective levels of aggregation and inactivation at 37°C as described in Example 9. All three immunotoxins were principally monomeric before incubation at 37°C. After one hour of incubation in PBS at 37°C, about half of B3V_H-B5V_L-PE38 and B3(Fv)PE38 V_L: M4L S7T were aggregated, whereas B3(Fv)-PE38 was about 75% aggregated. After 2 hours of incubation in PBS at 37°C, 60% of B3V_H-B5V_L-PE38 and B3(Fv)-PE38 V_L: M4L S7T were aggregated, and B3(Fv)-PE38 was >80% aggregated. After 4 hours of incubation in PBS at 37°C, about 80% B3V_H-B5V_L-PE38 and B3(Fv)-PE38 V_L: M4L S7T had aggregated, whereas B3(Fv)PE38 was almost completely aggregated.

The cytotoxic activities of these immunotoxins are shown in Figure 10. Following the 1 hour incubation at 37°C in PBS, B3(Fv)-PE38 had an IC_{50} of 8 ng/ml which is 25% of its cytotoxic activity before treatment. After 2 hours at 37°C, it had an IC_{50} of 200 ng/ml which is about 1% of its cytotoxic activity before treatment. Both B3V_H-B5V_L-PE38 and B3(Fv)-PE38: V_L M4L S7T cytotoxic activities after one hour at 37°C in PBS were similar to their pretreatment activities. After 2 hours they showed an IC_{50} of 3.5 ng/ml which is 12% of their cytotoxic activity before treatment.

The lower IC_{50} of the chimera and the mutant can be explained by the fact that both are more stable than the wild type B3(Fv)-PE38. This improved stability was evident from their slower aggregation and loss of cytotoxic activity upon incubation in PBS at 37°C. Very little B3(Fv)-PE38 monomer survives a 2 hours incubation, whereas the stabilized variants survive for a longer time. This correlates with the fact that while

B3(Fv)-PE38 cytotoxic activity is only slightly increased if A431 cells are exposed to it for 20 hours instead of two hours, whereas the stabilized variants show a 3 fold increase upon a 20 hour incubation when compared to a 2 hour incubation on A431 cells.

Site specific mutagenesis was used to identify which of the three V_L residues that differ between B3 and B5 was previously responsible for the stabilizing effect. Since B3V_H-B5V_L-PE38 and B3(Fv)-PE38: V_L M4L S7T (which differs from the chimera only at the fourth CDR1 residue) had identical characteristics in the assays, the CDR residue is not the stabilizing one. Analysis of B3(Fv)-PE38 derivatives carrying mutations V_L M4L or V_L S7T separately showed that replacing V_L methionine 4 with leucine stabilized the immunotoxin as much as the B3V_H-B5V_L-PE38 combination, whereas replacing V_L serine 7 with threonine had no stabilizing effect (data not shown).

A binding study using a BioCore instrument indicated that B3(Fv)-PE38: V_L M4L S7T has a similar off rate to that of B3(Fv)-PE38 (0.0023 and 0.0021, respectively) whereas the on rates differ (1150 and 984, respectively). The apparent K_d is 2.33×10^{-6} for B3(Fv)-PE38 and 1.84×10^{-6} for B3(Fv)-PE38: V_L M4L S7T. This data correlates with binding data obtained by competition with ¹²⁵I B3 IgG.

Example 13

Humanization of the B3(Fv) Antibody

B3(Fv) was humanized by a process of "framework exchange". As will be explained in detail below, the variable domains of the heavy and light chains were aligned with human antibody sequences and, by comparison of each domain with its best human homolog, framework residues that differed between the mouse B3 and its human homolog were identified. Eleven framework residues in V_H and eight in V_L were changed by site-specific mutagenesis to human residues and introduced simultaneously into a pre-assembled single-chain Fv (scFv) expression cassette.

A) Identification of Residues for Humanization

A structural model of B3(Fv) was constructed based on the crystal structure of the variable domains of monoclonal antibody McPC603 (Satow *et al.*, *J. Mol. Biol.*, 190: 593-604 (1986); Abola *et al.*, pp. 107-132 in *Crystallographic databases-information content*, Software Systems, Scientific Application, eds. Allen, Bergerhoff, & Sievers, Data Comm. of the Intl. Union of Crystallogr., Bonn (1987);

Protein Data bank Entry IMCP), which was modified and refined by an energy minimization algorithm using the program CHARMM (Brooks *et al.*, *J. Comput. Chem.*, 4: 187-217 (1983)) version 22. The construction of this refined model is described in detail elsewhere (Jung *et al.*, *Protein Structure Function and Genetics*, 19: 35-47 (1994)). The amino acid sequences of B3 V_H and V_L were independently aligned with all the human antibody sequences contained in the SWISS-PROT Data Base using the FASTA program (Release 27.0 10/93) (Devereux *et al.*, *Nucleic Acids Res.* 12: 387-395 (1984)).

The V_H of the human fetal immunoglobulin 56P1'CL (Schroeder *et al.*, *Science*, 238: 791-793 (1987)) had the highest overall sequence identity and had the highest identity in the framework regions. The alignment of B3 V_H with 59P1'CL V_H is shown in Figure 11(A). The V_L of the human IgM GM607 (Klobeck *et al.*, *Nature*, 309, 73-76 (1984)) (SWISSPROT file sw:kv2e-human) scored fourth in overall sequence identity (77.7%), but had the highest identity in the framework regions. The alignment of B3 V_L with GM603 V_L is shown in Figure 11(B). The amino acid residues that differ are identified in Figure 11 by vertical lines above the sequence. Based on experiments with B3(Fv)-PE38 mutants (Benhar, unpublished), and on the analysis of B3Fv using the structural model, it was decided to preserve the mouse residues at V_H positions 1, 3, 19, 24, 89, and 91 and V_L positions 2, 3 and 41 (Kabat, *et al. Sequences of proteins of immunological interest*. 5th edition. U.S. (1991); Figure 11). These residues are identified by asterisks in Figure 11(A) and 11(B), some of them are inter-domain residues and others are buried and therefore are not expected to be part of an immunogenic epitope (Padlan, *Mol. Immunol.* 28: 489-498 (1991) and Roguska *et al. Proc. Natl. Acad. Sci. USA*, 91: 969-973 (1994)).

The human antibodies chosen also had similarity to B3(Fv) in the sequence of the complementarity determining region loops (CDRs), and had the same CDR length which further indicates that they belong to a similar structural group, and possibly have a similar canonical structure of the CDR loops (Chothia *et al.*, *J. Biol. Chem.* 227: 799-817 (1992); Williams *et al.*, *Eur. J. Immunol.* 23: 1456-1461 (1993)).

B) Construction of plasmids expressing humanized variants of B3(Fv)PE38.

B3 V_H and V_L gene segments in plasmid pULI7 (Fig. 13(A)) encoding wild type B3(Fv)-PE38 was selected for modification via site-directed mutagenesis.

Uracil-containing single-stranded DNA was prepared by rescue of our F+ origin containing plasmids with an M13KO7 helper phage and was used as a template for site-specific mutagenesis (Kunkel, *Proc. Natl. Acad. Sci. USA*, 82: 488-492 (1985)). The complete nucleotide sequence of the gene encoding B3(Fv)-PE38 has been described
5 (Brinkmann, *et al.*, *Proc. Natl. Acad. Sci. USA*, 88: 8616-8620 (1991)). Mutagenic oligonucleotides used and the mutation, they are listed in Table 4.

B3 V_H and V_L gene segments in plasmid pULI7 (Fig. 12(A)), encoding wild type B3(Fv)-PE38, were independently humanized by site specific mutagenesis. A set of four oligonucleotides was used to simultaneously introduce the mutations into each
10 segment, with most of the oligonucleotides changing more than one mouse to human codon. In B3 V_H, the mutations introduced were L11V and G16R, T40A, E42G and R44G, A74S and R75K, S82aN, R82bS, K83R and S84A. The resulting plasmid was pB3HUMV_H-V_L-PE38 (Fig. 12(B)). In B3 V_L, the mutations introduced were S14T, L15P, D17E and Q18P, K45Q, L83V, SLOOQ and L104V. The resulting plasmid,
15 pB3HUMV_H-V_L-PE38 (Fig. 12(C)) was used as a template for a second mutagenesis with the combined heavy chain mutagenic oligonucleotides generating plasmid pHUMV_H-HUMV_L-PE38 (Fig. 12(D)), which encodes the humanized B3(Fv) single-chain immunotoxin. The residues that were mutated are identified in Fig. 11 by their numbers.

20 **C) Expression and Purification of Recombinant Proteins**

Expression plasmids encoding B3(Fv)-PE38 or its humanized derivatives were introduced into *E. coli* strain BL21 (ΔDE3) (Studier *et al.* *J. Mol. Biol.* 189, 113-130 (1986)) and the recombinant proteins were expressed as inclusion bodies as described in Example 6. The single-chain immunotoxins were obtained by solubilization
25 and refolding of inclusion body protein as described and subsequently purified as described in Example 6. Typically, the monomeric proteins were obtained at over 95% purity, as determined by non-reducing SDS-PAGE of the product.

Table 4. Oligonucleotides utilized for site directed mutagenesis and the mutations they introduced. Restriction sites which were introduced into these oligonucleotides to facilitate identification of mutated clones are underlined.

Primer	Sequence	Mutation	Seq. ID. No.
1	5'-GGAGAGTTTCAGGGAGCG <u>CCCGGG</u> GTGC ACGACGCCTCCCCC-3'	V _H : L11V; G16R	23
2	5'-TGCGACCCACTCCAGGCCCTTG <u>CCCGGG</u> GCCTGG CGAACCCAATA-3'	V _H : T40A; E42G; R44G	24
3	5'-GAGGGTGTCTTGCTATTGTCTCTAGAG ATGGTGAACCG-3'	V _H : A74S; R75K	25
4	5'-TATGGCTGTGTCTCGGCGCGCAGGCTG TTCATTTCAGGTA-3'	V _H : S82aN; R82bS; K83R; S84A	26
5	5'-GCAAGAGATGGAGCCGGCTCT <u>CCCGGG</u> GTGACAGGTAAACTCAA-3'	V _L : S14T; L15P; D17E; Q18P	27
6	5'-AACTTTGTAGATCAGCAGCTGTGGAGAC TGGGCTGG-3'	VL: K45Q	28
7	5'-GCAGTAATAAACTCCGACGTCCTCAGCC TCCAC-3'	V _L : L83V/	29
8	5'-GGAAGCTTTAATTTGACCTTGGTACCC TGGCCGAACGTGAATGG-3'	V _L : S100Q; L104	30

D) Cytotoxicity and Binding Affinity of Humanized B3(Fv)-PE38

The ADP-ribosylation activity of each immunotoxin was tested, to verify that they are of equal enzymatic activity. As shown in Figure 13(A), B3(Fv)-PE38 and the humanized variants had similar ADP-ribosylation activities.

The cytotoxic activity of B3(Fv)-PE38 and of its humanized variants was measured by incubating various human carcinoma cell lines with serial dilutions of the immunotoxin, and measuring the incorporation of [³H]-leucine as described in Example 8. As shown in Figure 13(B), B3(Fv)-PE38 had an IC₅₀ of 1.8 ng/ml on A431 cells which express high levels of the B3 antigen. The variant B3V_H-HUMV_L-PE38 (HUM_L) had a similar cytotoxic activity, while B3HUMV_H-V_L-PE38 (HUM_H) and B3-HUMV_H-HUMV_L-PE38 (HUM_{H+L}) had IC₅₀s of about 4.4 ng/ml.

To check whether humanizing B3(Fv)-PE38 caused a change in antigen specificity, the same cytotoxic assay was done on additional cell lines. As shown in

Table 5, B3(Fv)-PE38 and all the humanized variants had the same spectrum of recognition of the cell lines used. These cell lines differ in their level of B3 antigen expression (Brinkmann *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 7538-7542 (1993). This result indicates that the antigen binding specificity of the B3(Fv) was not altered by the humanizing process.

The specific antigen binding affinity of the B3(Fv) immunotoxins was further analyzed by determination of the binding affinity of B3(Fv)-PE38 and the humanized variants to B3 antigen bearing cells by a competition assay, in which increasing concentrations of each immunotoxin were used to compete for the binding of [¹²⁵I]-B3 IgG to A431 cells at 4°C. As shown in Fig. 13(C), both B3(Fv)-PE38 and HUM_L blocked the binding of [¹²⁵I]-B3 antibody to A431 cells by 50% at 1-2 μM. However, HUM_H and HUM2_{H+L} had a lower affinity, and competed by 50% at 4-5 μM. The results from the cytotoxicity and the binding assays indicate that the humanized B3(Fv) suffered a 2-3 fold loss in antigen binding affinity and that the damaging mutation probably resides in the V_H segment.

Table 5. Cytotoxicity of recombinant B3(Fv)PE38 and its humanized variants toward various cell lines.

C ll Line	Source	B3 antigen expression	B3(Fv)PE38	HUM _L	HUM _H	HUM _{H+L}	HUMFv
A431	Epidermoid carcinoma	+++	1.8	1.9	4.4	4.2	2.2
MCF7	Breast carcinoma	+++	4.0	3.8	8.2	8.5	4.2
LnCap	Prostate carcinoma	+	35	30	110	90	300
KB 3-1	Cervical carcinoma	-	>1000	>1000	>1000	>1000	>1000
HUT102	T-cell leukemia	-	>1000	>1000	>1000	>1000	>1000
L929	Mouse fibroblast	-	>1000	>1000	>1000	>1000	>1000

Cytotoxicity data are given as IC₅₀ values, the concentration of immunotoxin that causes a 50% inhibition of protein synthesis following its incubation on the cells for 20 hours. Expression level estimation of the B3 antigen is based on immunofluorescence. +++, strong; + weak; -, not detected. The immunotoxins used are B3(Fv)PE38, B3V_H-HUMV_L-PE38 (HUM_L), B3HUMV_H-V_L-PE38 (HUM_H), B3HUMV_H-HUMV_L-PE38 (HUM_{H+L}), HUMB3(Fv)-PE38 (HUMFv). All cell lines except L929 are of human origin.

E) Back-Mutating V_H Residue 82b to Restore Activity

To restore the reduced activity of HUM_{H+L}, some mutated forms which were partially humanized in the V_H region were tested for cytotoxic activity. It was found that a mutant of B3(Fv)-PE38 in which the V_L was wild type and V_H residues at positions 74, 75 or 83 were humanized, did not lose cytotoxic activity, while a derivative in which residues 74, 75 and 82a, 82b, 83, and 84 were humanized was about 5-6 fold less active. In the B3 V_H structural group (mouse III(A)) residue 82a is most commonly asparagine, as it is in HUM_{H+L}. However, serine is never found at position 82b. Furthermore, the original residue at position 82b, arginine, is acceptable in human IgGs. Therefore, residue 82b in HUM_{H+L} was mutated to arginine by site-specific mutagenesis. The resulting molecule is named HUMB3(Fv)-PE38. The protein was purified to near homogeneity and was subjected to activity and binding assays. As shown in Figure 13(A), its ADP-ribosylation activity did not differ significantly from that of the other immunotoxins. Moreover, as shown in Figures 13(B), 13(C), and Table 5, its cytotoxic and antigen binding activities were improved relative to HUM_{H+L} and were similar to those of the original B3(Fv)-PE38 immunotoxin.

Example 17**Reactivity with Sera from Monkeys Immunized with B3(Fv)-PE38**

Sera obtained from monkeys that had been immunized with B3(Fv)-PE38 contain antibodies to PE38 as well as a lower reactivity with B3Fv. To assess the success of humanizing B3Fv, sera from four Cynomolgus monkeys containing specific anti B3(Fv)-PE38 titers (*Id.*) were pooled and used in an ELISA assay on plates that were coated with B3(Fv)-PE38, HUM_{H+L}, or HUMFv (B3HUMFv). An excess of PE38 was included as a competitor to preadsorb reactivity which is directed against the toxin moiety of the molecule. As shown in Figure 14, the anti B3(Fv)-PE38 sera had a weaker reaction against both HUM_{H+L} and HUMFv than with the wild-type B3(Fv)PE38, indicating that primate B3(Fv) epitope(s) were missing in the humanized variants.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

Example 18**The V_H Tyrosine to Serine Mutation Improves the Affinity of Anti-Le^Y Carbohydrate Antibodies**

When comparing the amino acid sequence of B3 to the conserved framework sequences of other antibodies (Kabat *et al.* (1991) *supra.*) it was observed that a tyrosine at position V_H 95 was exchanged to serine, which is very unusual. To investigate the significance of this exchange, this residue was mutated to tyrosine or to phenylalanine, which are the amino acids that are mostly present at this position in other antibodies. It was found, that both exchanges reduced the affinity of B3(Fv) compared to the molecule with the original serine approximately 10 fold. Thus, the serine is important for high affinity binding. This was unexpected and surprising, because position V_H 95 is in the V_H-V_L interface, and thus neither in the binding region nor close to it (*see*, commonly assigned U.S. Patent Application Serial No. 08/077,252 filed on June 14, 1993). This excludes the possibility that a direct (contact) effect of the serine, influences binding. The most likely explanation for the effect of the serine is, that, because it is positioned in the V_H-V_L interface and slightly destabilizes the interface contacts it enables a movement of V_H relative to V_L, mediating a so called "induced fit" antibody binding mode (Rini *et al.*, *Science*, 255: 959-965 (1992) and Stanfield *et al. Structure*, 1: 83-93 (1993)). To analyze whether the serine mutation" at V_H 95, which was found in B3(FV) increases only the affinity of B3(Fv) or if it can (possibly by mediating induced fit) also increase the affinity of other Le^Y binding antibodies, we introduced this mutation into B5(Fv). B5 Fv binds like B3 Le^Y but contains a different antibody binding site, and in addition contains the "conserved" tyrosine in position V_H 95. Exchange of this tyrosine to serine was done by site directed mutagenesis (Kunkel, *et al.* 1985) *supra.*). The resulting B5(Fv)-ser V_H 95 mutant molecule (designated B5(Fv): V_H Y95S) showed 4-fold increase in specific binding activity as analyzed by cytotoxicity assays, from which the (relative) affinities can be deduced. Thus, the V_H tyrosine to serine 95 mutation not only increases the affinity of B3(Fv) but also of one other Le^Y binding antibody and probably others. Since the mechanism by which the ser 95 causes increased affinity is most likely facilitation of induced fit binding, it is expected that this mutation can also increase the affinity of certain other antibodies, in cases where induced fit will generate increased interactions between the antibody and the antigen.

Table 5. Relative binding affinity of single chain fusion proteins with substitutions at V_H position 95.

Single Chain Antibody	Amino Acid at Position 95 of V _H	Percentage Binding Affinity*
B3(Fv)	tyrosine	10%
B3(Fv)	serine	100%
B5(Fv)	tyrosine	25%
B5(Fv)	serine	100%

* Best binding affinity was set to 100%.

SEQUENCE LISTING

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FUSION PROTEINS, AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 53
- (iv) CORRESPONDENCE ADDRESS:
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 - (D) STATE: California
 - (E) COUNTRY: US
 - (F) ZIP: 94105-1493
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/767,331
 - (B) FILING DATE: 30-SEP-1991
- (viii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/596,289
 - (B) FILING DATE: 12-OCT-1990
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 44 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

(A) NAME/KEY: misc feature

(B) LOCATION: 1..44

(D) OTHER INFORMATION: /standard_name= "Heavy chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1.

TAACTAGGAT CCGTCCATAT GGATGTGAAG CTGGTGGAGT CTGG

44

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

(A) NAME/KEY: misc feature

(B) LOCATION: 1..39

(D) OTHER INFORMATION: /standard_name= "Heavy chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGATAGACT GATGGGGATC CGCCTCCGCC TGAGGAGAC

39

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

(A) NAME/KEY: misc feature

(B) LOCATION: 1..39

(D) OTHER INFORMATION: /standard_name= "Heavy chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GATATACATA TGGAGGTGCA GCTGGTGGAA TCTGGAGGA

39

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..39
- (D) OTHER INFORMATION: /standard_name= "Heavy chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATATACATA TGGAGGTGAA GCTGGTGGAA TCTGGAGG..

39

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..24
- (D) OTHER INFORMATION: /standard_name= "Heavy chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGCAGATCCA GGGGCCAGTG GATA

24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..27
- (D) OTHER INFORMATION: /standard_name= "Heavy chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACCGGATCCG CCTGCAGAGA CAGTGAC

27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc featur
- (B) LOCATION: 1..33
- (D) OTHER INFORMATION: /standard_name = "H avy chain primer B5HRr4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCGGATCCG CCTCCGCCTG AGGAGACAGT GAS

33

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..70
- (D) OTHER INFORMATION: /standard_name= "Light chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCTCCAAGC TTGGGGATCC GGTGGTGGCG GATCTGGAGG TGGCGGAAGC GATGTGCTGA
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60

70

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..42
- (D) OTHER INFORMATION: /standard_name= "Light chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGTTGGTGCA GCATCAAAAG CTTTKAKYTC CAGCTTKGTS CC

42

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..28
- (D) OTHER INFORMATION: /standard_name= "Light chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTGGGGATCC GGTGGTGGCG GATCTGGA

28

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..40
- (D) OTHER INFORMATION: /standard_name= "Light chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCGGGAATT CATTATTTAA TTTCCAGCTT TGTCCCCGAC

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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..30
- (D) OTHER INFORMATION: /standard_name= "Light chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTGGCGGAA GCGATGTTGT GATGACCCAA

30

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc_featur

- (B) LOCATION: 1..30
- (D) OTHER INFORMATION: /standard_name= "Light chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGTGGCGGAA GCGATGTTTT GTTGACCCAA

30

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..24
- (D) OTHER INFORMATION: /standard_name= "Light chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGGTGGGAAG ATGGATACAG TTGG

24

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..24
- (D) OTHER INFORMATION: /standard_name= "Light chain primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGAAGCTTTC AGCTCCAGCT TGGT

24

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..23
- (D) OTHER INFORMATION: /standard_name= "Light chain"

primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGAAGCTTTA TTTCCAACCTT TCT

23

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 357 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..357
- (D) OTHER INFORMATION: /standard_name= "B3
variable heavy chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GATGTGAAGC TGGTGGAGTC TGGGGGAGGC TTAGTGCAGC CTGGAGGGTC CCTGAACTC	60
TCCTGTGCAA CCTCTGGATT CACTTTCAGT GACTATTACA TGTATTGGGT TCGCCAGACT	120
CCAGAGAAGA GGCTGGAGTG GGTGCGATAC ATTAGTAATG ATGATAGTTC CGCCGCTTAT	180
TCAGACACTG TAAAGGGCCG GTTCACCATC TCCAGAGACA ATGCCAGGAA CACCCTCTAC	240
CTGCAAATGA GCCGTCTGAA GTCTGAGGAC ACAGCCATAT ATTCCTGTGC AAGAGGACTG	300
GCCTGGGGAG CCTGGTTTGC TTACTGGGGC CAAGGGACTC TGGTCACTGT CTCCTCA	357

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 336 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..336
- (D) OTHER INFORMATION: /standard_name= "B3
variable light chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GATGTGCTGA TGACCCAGTC TCCATTGAGT TTACCTGTCA GTCTTGGAGA TCAAGCCTCC	60
ATCTCTTGCA GATCTA TCA GATCATTGTA CATAGTAATG AAACACCTA TTTAGAATGG	120
TACCTGCAGA AACCAGGCCA GTCTCCAAAG CTCCTGATCT ACAAAGTTTC CAACCGATTT	180
TCTGGGGTCC CAGACAGGTT CAGTGGCAGT GGATCAGGGA CAGATTTTAC ACTCAAGATC	240
AGCAGAGTGG AGGCTGAGGA TCTGGGAGTT TATTACTGCT TTCAAGGTTT ACATGTTCCA	300

TTCACGTTTCG GCTCGGGGAC AAAGCTGGAA ATTAAA

336

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 363 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: misc feature
 (B) LOCATION: 1..363
 (D) OTHER INFORMATION: /standard_name= "B1
 variable heavy chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GAGGTGCAGC TGGTGAATC TGGAGGAGGC TTAGTGAAGC CTGGAGGGTC CCTGAAACTC	60
TCCTGTGCAG CCTCTGGATT CATTTTCAGT GACAATTACA TGTATTGGGT TCGCCAGACT	120
CCGGAGAAGA GGCTGGAGTG GGTGCAACC ATTAGTGATG GTGGCACTTA TATCGACTAT	180
TCAGACAGTG TGAAGGGGCG ATTCACCATC TCCAGAGACA ATGCCAAGAA TAATCTGTAC	240
TTGCAAATGA GCAGTCTGAG GTCTGAGGAC ACAGGCATGT ATTATTGTGG AAGGAGTCCG	300
ATCTACTATG ATTACGCCCC GTTACTTAC TGGGGCCAAG GGA CTCTGGT CACTGTCTCT	360
GCA	363

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 336 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: misc feature
 (B) LOCATION: 1..336
 (D) OTHER INFORMATION: /standard_name= "B1
 variable light chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GATGTTGTGA TGACCCAGAC TCCACTCTCC CTGCCTGTCA GTCTTGGAGA TCAAGCCTCC	60
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TTCCTGCAGA AGCCTGGCCA GTCTCCAACG CTCCTGATCT ACAAAGTTTC CAACCGATTT	180
TCTGGGGTCC CAGACAGGTT CAGTGGCAGT GGATCAGGGA CAGATTTTCAT ACTCAAGATC	240
AGCAGAGTGG AGGCTGAGGA TCTGGGAGTT TATTTCTGCT CTCAAAGTAC ACATGTTCCG	300
CTCACGTTTCG GTGCTGGGAC CAAGCTGGAG CTGAAA	336

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 357 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..357
- (D) OTHER INFORMATION: /standard_name= "B5
variable heavy chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

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TCCTGTGCAA CCTCTGGATT TACTTTCAGT GACTATTACA TGTATTGGGT TCGCCAGACT	120
CCAGAGAAGA GGCTGGAGTG GGTCGCATAC ATTAGTAATG GTGGTGGTAG CACCTATTAT	180
CCAGACACTG TAAAGGGCCG ATTCACCATC TCCAGAGACA ACGCCAAGAA CACCCTGTAC	240
CTGCAGATGA GCCGTCTGAA GTCTGAGGAC ACAGCCATGT ATTACTGTGC AAGGGGGCTC	300
TCTGATGGTT CCTGGTTTGC TTACTGGGGC CAAGGGACTC TGGTCACTGT CTCCTCA	357

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 336 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..336
- (D) OTHER INFORMATION: /standard_name= "B5
variable light chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATGTTTTGT TGACCCAAAC TCCACTCTCC CTGCCTGTCA GTCTTGGAGA TCAAGCCTCT	60
ATTTCTTGTA GATCTAGTCA GAGCATTGTA CATAGTAATG GAAACACCTA TTTAGAATGG	120
TACCTGCAGA AACCAGGCCA GTCTCCAAAG CTCCTGATCT ACAAAGTTTC CAACCGATTT	180
TCTGGGGTCC CAGACAGGTT CAGTGGCAGT GGATCAGGGA CAGATTTCAC ACTCAAGATC	240
AGCAGAGTGG AGGCTGAGGA TCTGGGAGTT TATTACTGCT TTCAAGGTTC ACATGTTCCA	300
TTCACGTTTCG GCTCGGGGAC AAAGTTGGAA ATTAA	336

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

(A) NAME/KEY: misc feature
(B) LOCATION: 1..42
(D) OTHER INFORMATION: /label= Primer_1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGAGAGTTTC AGGGAGCGCC CGGGGTGCAC GACGCCTCCC CC

42

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

(A) NAME/KEY: misc feature
(B) LOCATION: 1..45
(D) OTHER INFORMATION: /label= Primer_2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TGCGACCCAC TCCAGGCCCT TGCCCGGGC CTGGCGAACC CAATA

45

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

(A) NAME/KEY: misc feature
(B) LOCATION: 1..39
(D) OTHER INFORMATION: /label= Primer_3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GAGGGTGTTT TTGCTATTGT CTCTAGAGAT GGTGAACCG

39

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..42
- (D) OTHER INFORMATION: /label= Primer_4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TATGGCTGTG TCCTCGGCGC GCAGGCTGTT CATTTGCAGG TA

42

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..45
- (D) OTHER INFORMATION: /label= Primer_5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GCAAGAGATG GAGGCCGGCT CTCCGGGGT GACAGGTAAA CTCAA

45

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..36
- (D) OTHER INFORMATION: /label= Primer_6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AACTTTGTAG ATCAGCAGCT GTGGAGACTG GGCTGG

36

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (prim r)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..33
- (D) OTHER INFORMATION: /label= Prim r_7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GCAGTAATAA ACTCCGACGT CCTCAGCCTC CAC

33

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..45
- (D) OTHER INFORMATION: /label= Primer_8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGAAGCTTTA ATTCGACCT TGGTACCCTG GCCGAACGTG AATGG

45

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 741 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..741
- (D) OTHER INFORMATION: /note= "Sequence encoding humanized B3(Fv)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

ATGGATGTGA AGCTGGTGGG GTCTGGGGGA GGCGTCGTGC AGCCCGGGCG CTCCCTGAAA	60
CTCTCCTGTG CAACCTCTGG ATTCACCTTC AGTGACTATT ACATGTATTG GGTTCGCCAG	120
GCCCCGGGCA AGGGCCTGGA GTGGGTCGCA TACATTAGTA ATGATGATAG TTCCGCCGCT	180
TATTCAGACA CTGTAAAGGG CCGGTTACAC ATCTCTAGAG ACAATAGCAA GAACACCCTC	240
TACCTGCAAA TGAACCGTCT GCGCGCCGAG GACACAGCCA TATATTCCTG TGCAAGAGGA	300
CTGGCCTGGG GAGCCTGGTT TGCTTACTGG GGCCAAGGGA CTCTGGTCAC TGTCTCCTCA	360
GGCGGAGGCG GATCCGGTGG TGGCGGATCT GGAGGTGGCG GAAGCGATGT GCTGATGACC	420
CAGTCTCCAT TGAGTTTACC TGTACCCCGG GGAGAGCCGG CCTCCATCTC TTGCAGATCT	480
AGTCAGATCA TTGTACATAG TAATGGAAC ACCTATTTAG AATGGTACCT GCAGAAACCA	540
GGCCAGTCTC CACAGCTGCT GATCTACAAA GTTTCCAACC GATTTTCTGG GGTCCCAGAC	600
AGGTTCAAGT GCAGTGGATC AGGGACAGAT TTCACACTCA AGATCAGCAG AGTGGAGGCT	660

GAGGACGTCG GAGTTTATTA CTGCTTTCAA GGTTCACAT TTCCATTAC GTTCGGCCAG 720
 GGTACCAAGG TCGAAATTAA A 741

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..15
- (D) OTHER INFORMATION: /label= LINKER

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
 1 5 10 15

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 772 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 27..767

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TTTAACTTTA AGAAGGAGAT ATACAT ATG GAT GTG AAG CTG GTG GAG TCT GCG 53
 Met Asp Val Lys Leu Val Glu Ser Gly
 1 5

GGA GGC TTA GTG CAG CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA ACC 101
 Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Thr
 10 15 20 25

TCT GGA TTC ACT TTC AGT GAC TAT TAC ATG TAT TGG GTT CGC CAG ACT 149
 Ser Gly Phe Thr Phe Ser Asp Tyr Tyr Met Tyr Trp Val Arg Gln Thr
 30 35 40

CCA GAG AAG AGG CTG GAG TGG GTC GCA TAC ATT AGT AAT GAT GAT AGT 197
 Pro Glu Lys Arg Leu Glu Trp Val Ala Tyr Ile Ser Asn Asp Ser
 45 50 55

TCC GCC GCT TAT TCA GAC ACT GTA AAG GGC CGG TTC ACC ATC TCC AGA 245
 Ser Ala Ala Tyr S r Asp Thr Val Lys Gly Arg Phe Thr Ile Ser Arg
 60 65 70

GAC AAT GCC AGG AAC ACC CTC TAC CTG CAA ATG AGC CGT CTG AAG TCT 293
 Asp Asn Ala Arg Asn Thr L u Tyr Leu Gln Met S r Arg Leu Lys Ser
 75 80 85

GAG GAC ACA GCC ATA TAT TCC TGT GCA AGA GGA CTG GCC TGG GGA GCC 341

lu	Asp	Thr	Ala	Ile	Tyr	Ser	Cys	Ala	Arg	Gly	Leu	Ala	Trp	ly	Ala		
90					95					100					105		
TGG	TTT	CT	TAC	TGG	GGC	CAA	GG	ACT	CTG	GTC	ACT	GTC	TCC	TCA	GGC		389
Trp	Phe	Ala	Tyr	Trp	Gly	Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Gly		
				110					115					120			
GGA	GGC	GGA	TCC	GGT	GGT	GGC	GGA	TCT	GGA	GGT	GGC	GGA	AGC	GAT	GTC		437
Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Asp	Val		
			125					130					135				
CTG	ATG	ACC	CAG	TCT	CCA	TTG	AGT	TTA	CCT	GTC	AGT	CTT	GGA	GAT	CAA		485
Leu	Met	Thr	Gln	Ser	Pro	Leu	Ser	Leu	Pro	Val	Ser	Leu	Gly	Asp	Gln		
		140					145					150					
GCC	TCC	ATC	TCT	TGC	AGA	TCT	AGT	CAG	ATC	ATT	GTA	CAT	AGT	AAT	GGA		533
Ala	Ser	Ile	Ser	Cys	Arg	Ser	Ser	Gln	Ile	Ile	Val	His	Ser	Asn	Gly		
		155				160					165						
AAC	ACC	TAT	TTA	GAA	TGG	TAC	CTG	CAG	AAA	CCA	GGC	CAG	TCT	CCA	AAG		581
Asn	Thr	Tyr	Leu	Glu	Trp	Tyr	Leu	Gln	Lys	Pro	Gly	Gln	Ser	Pro	Lys		
170				175					180						185		
CTC	CTG	ATC	TAC	AAA	GTT	TCC	AAC	CGA	TTT	TCT	GGG	GTC	CCA	GAC	AGG		629
Leu	Leu	Ile	Tyr	Lys	Val	Ser	Asn	Arg	Phe	Ser	Gly	Val	Pro	Asp	Arg		
				190				195					200				
TTC	AGT	GGC	AGT	GGA	TCA	GGG	ACA	GAT	TTC	ACA	CTC	AAG	ATC	ACC	AGA		677
Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Lys	Ile	Ser	Arg		
			205					210					215				
GTG	GAG	GCT	GAG	GAT	CTG	GGA	GTT	TAT	TAC	TGC	TTT	CAA	GGT	TCA	CAT		725
Val	Glu	Ala	Glu	Asp	Leu	Gly	Val	Tyr	Tyr	Cys	Phe	Gln	Gly	Ser	His		
		220				225						230					
GTT	CCA	TTC	ACG	TTC	GGC	TCG	GGG	ACA	AAG	CTG	GAA	ATT	AAA				767
Val	Pro	Phe	Thr	Phe	Gly	Ser	Gly	Thr	Lys	Leu	Glu	Ile	Lys				
		235				240					245						
GCTTT																	772

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 247 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Met	Asp	Val	Lys	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly		
1				5					10					15			
Gly	Ser	Leu	Lys	Leu	Ser	Cys	Ala	Thr	Ser	Gly	Phe	Thr	Phe	Ser	Asp		
		20						25					30				
Tyr	Tyr	Met	Tyr	Trp	Val	Arg	Gln	Thr	Pro	Glu	Lys	Arg	Leu	Glu	Trp		
		35				40					45						
Val	Ala	Tyr	Ile	Ser	Asn	Asp	Asp	Ser	Ser	Ala	Ala	Tyr	Ser	Asp	Thr		
		50				55					60						
Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Arg	Asn	Thr	Leu		
		65				70				75					80		

[illegible]

(2) INFORMATION FOR SEQ ID NO:35:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 3..47

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CA CAT GTT CCA TTC ACG TTC GGC TCG GGG ACA AAG CTG GAA ATT AAA
His Val Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
1 5 10 15

47

TAATGAATTC C

58

(2) INFORMATION FOR SEQ ID NO:36:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
(B) TYPE: amin acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

His Val Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CTCTCCCTG

9

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TTGAGTTTA

9

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCAGTCTCCA CTCTCC

16

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID N :40:

Met lu Val Gln Leu Val Glu Ser Gly ly
1 5 10

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Met Glu Val Lys Leu Val Glu Ser Gly Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Gly Gly Gly Ser Asp Val Val Met Thr Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Gly Gly Gly Ser Asp Val Leu Leu Thr Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: singl

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Ser Gly Gly Pro lu Gly Gly Ser
1 5

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 119 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..119
- (D) OTHER INFORMATION: /note= "B3 VH region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Asp Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Lys Leu Ser Cys Ala Thr Ser Gly Phe Thr Phe Ser Asp Tyr
20 25 30

Tyr Met Tyr Trp Val Arg Gln Thr Pro Glu Lys Arg Leu Glu Trp Val
35 40 45

Ala Tyr Ile Ser Asn Asp Asp Ser Ser Ala Ala Tyr Ser Asp Thr Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Arg Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Ser Arg Leu Lys Ser Glu Asp Thr Ala Ile Tyr Ser Cys
85 90 95

Ala Arg Gly Leu Ala Trp Gly Ala Trp Phe Ala Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 119 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..119
- (D) OTHER INFORMATION: /note= "Human f tal immunoglobulin
56Pl'CL VH region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Gln Val Glu Leu Val Glu Ser Gly Gly Gly Val Val Gln Pr Gly Arg
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser S r Tyr
 20 25 30
 Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 Ala Val Ile Ser Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Arg Ser Ala Arg Thr Tyr Tyr Phe Asp Tyr Trp Gly Gln Gly
 100 105 110
 Thr Leu Val Thr Val Ser Ser
 115

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 119 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..119
- (D) OTHER INFORMATION: /note= "Humanized B3 VH region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Asp Val Lys Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1 5 10 15
 Ser Leu Lys Leu Ser Cys Ala Thr Ser Gly Phe Thr Phe Ser Asp Tyr
 20 25 30
 Tyr Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 Ala Tyr Ile Ser Asn Asp Asp Ser Ser Ala Ala Tyr Ser Asp Thr Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Asn Arg Leu Arg Ala Glu Asp Thr Ala Ile Tyr Ser Cys
 85 90 95
 Ala Arg Gly Leu Ala Trp Gly Ala Trp Phe Ala Tyr Trp Gly Gln ly
 100 105 110
 Thr Leu Val Thr Val Ser Ser
 115

(2) INFORMATION FOR SEQ ID N :48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 112 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..112
- (D) OTHER INFORMATION: /note= "B3 VL region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

```

Asp Val Leu Met Thr Gln Ser Pro Leu Ser Leu Pro Val Ser Leu Gly
1           5           10           15
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ile Ile Val His Ser
20           25           30
Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35           40           45
Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
50           55           60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65           70           75           80
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Gly
85           90           95
Ser His Val Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100          105          110

```

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 112 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..112
- (D) OTHER INFORMATION: /note= "Human IgM GM607 VL region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

```

Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
1           5           10           15
Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu His Ser
20           25           30
Asn Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys Pro Gln Gln Ser
35           40           45

```

Pro Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala S r Gly Val Pro
 50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Il
 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Gly
 85 90 95

Leu Gln Thr Pro Gln Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
 100 105 110

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 112 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..112
- (D) OTHER INFORMATION: /note= "Humanized B3 VL region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Asp Val Leu Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
 1 5 10 15

Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ile Ile Val His Ser
 20 25 30

Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
 35 40 45

Pro Gln Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
 50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Gly
 85 90 95

Ser His Val Pro Phe Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
 100 105 110

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Lys Asp Glu Leu
1

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Arg Glu Asp Leu
1

(2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Arg Glu Asp Leu Leu
1 5

WHAT IS CLAIMED IS:

- 1 1. A recombinant DNA molecule that encodes a single chain fusion
2 protein, said recombinant DNA molecule comprising:
 - 3 a) a DNA sequence that encodes the Fv region of both the light
4 and heavy chains of an antibody; and
 - 5 b) a DNA sequence that encodes an effector molecule; wherein
6 said fusion protein has the binding specificity of a monoclonal antibody selected from the
7 group consisting of B1, B3, and B5.
- 1 2. The recombinant DNA molecule of claim 1, wherein said effector
2 molecule is a *Pseudomonas* exotoxin.
- 1 3. The recombinant DNA molecule of claim 2, wherein said effector
2 molecule is selected from the group consisting of PE38, PE40, PE38KDEL, PE38REDL.
- 1 4. The recombinant DNA molecule of claim 1, wherein said antibody
2 is B1.
- 1 5. The recombinant DNA molecule of claim 1, wherein said antibody
2 is B3.
- 1 6. The recombinant DNA molecule of claim 1, wherein said antibody
2 is B5.
- 1 7. The recombinant DNA molecule of claim 1, wherein said molecule
2 encodes a fusion protein selected from the group consisting of B1(Fv)-PE38, B3(Fv)-
3 PE38, B3(Fv)-PE40, B3(Fv)-PE38KDEL, B3(Fv)-PE38REDL, and B5(Fv)-PE38.
- 1 8. A recombinantly produced single chain fusion protein comprising:
 - 2 a) the Fv region of both the light and heavy chains of an antibody;
 - 3 and b) an effector molecule;

4 wherein said Fv region and said effector molecule are recombinantly fused to form a
5 single chain molecule that has the binding specificity a monoclonal antibody selected
6 from the group consisting of B1, B3, and B5.

1 9. The fusion protein of claim 8, wherein said effector molecule is a
2 *Pseudomonas* exotoxin.

1 10. The fusion protein of claim 9, wherein said effector molecule is
2 selected from the group consisting of PE38, PE40, PE38KDEL and PE38REDL.

1 11. The fusion protein of claim 8, wherein said antibody is B1.

1 12. The fusion protein of claim 8, wherein said antibody is B3.

1 13. The fusion protein of claim 8, wherein said antibody is B5.

1 14. The fusion protein of claim 8, wherein said fusion protein is
2 selected from the group consisting of B1(Fv)-PE38, B3(Fv)-PE38, B3(Fv)-PE40,
3 B3(Fv)-PE38KDEL, B3(Fv)-PE38REDL, and B5(Fv)-PE38.

1 15. A recombinant DNA molecule that encodes a single chain antibody,
2 said recombinant DNA molecule comprising a DNA sequence that encodes the Fv region
3 of both the light and heavy chains of an antibody; wherein said DNA sequences are
4 recombinantly fused to form a single molecule and wherein said fusion protein has the
5 binding specificity of a monoclonal antibody selected from the group consisting of B1,
6 B3, and B5.

1 16. The recombinant DNA molecule of claim 15, wherein said antibody
2 is B1.

1 17. The recombinant DNA molecule of claim 15, wherein said antibody
2 is B3.

1 18. The recombinant DNA molecule of claim 15, wherein said antibody
2 is B5.

1 19. A recombinantly produced single chain antibody comprising an Fv
2 region of both a light and a heavy chain of an antibody where said light and heavy chains
3 are recombinantly fused to form a single molecule which has the binding specificity of a
4 monoclonal antibody selected from the group consisting of B1, B3, and B5.

1 20. The single chain antibody of claim 19, wherein said antibody is B1.

1 21. The single chain antibody of claim 19, wherein said antibody is B3.

1 22. The single chain antibody of claim 19, wherein said antibody is B5.

1 23. A pharmaceutical composition comprising a recombinantly
2 produced single chain fusion protein in a concentration sufficient to inhibit tumor cell
3 growth, together with a pharmaceutically acceptable carrier wherein said fusion protein
4 comprises:

5 a) a single-chain Fv region of an antibody, said Fv region
6 comprising the V_H and V_L regions of said antibody; and

7 b) an effector molecule;
8 wherein said Fv region and said effector molecule are recombinantly fused to form a
9 single molecule that has the binding specificity of a monoclonal antibody selected from
10 the group consisting of B1, B3, and B5.

1 24. The composition of claim 23, wherein said effector molecule is a
2 *Pseudomonas* exotoxin.

1 25. The composition of claim 24, wherein said effector molecule is
2 selected from the group consisting of PE38, PE40, PE38KDEL, PE38REDL.

1 26. The composition of claim 25 wherein said antibody is B1.

1 27. The composition of claim 25 wherein said antibody is B3.

1 28. The composition of claim 25 wherein said antibody is B5.

1 29. A method of detecting the presence or absence of a cell bearing a
2 Lewis^Y carbohydrate antigen in a patient, said method comprising the steps of:
3 a) removing a tissue or fluid sample from said patient;
4 b) adding an antibody to said sample wherein said antibody
5 comprises the Fv region of both the light and heavy chains of an antibody and said
6 antibody has the binding specificity of a monoclonal antibody selected from the group
7 consisting of B1, B3, and B5; and
8 c) detecting for the presence or absence of a binding complex
9 between the antibody and the antigen.

1 30. A method of killing or inhibiting the growth of cells bearing a
2 Lewis^Y antigen in a patient, said method comprising administering to the patient a
3 pharmaceutical composition in an amount sufficient to kill or inhibit the growth of said
4 cells, said composition comprising:
5 a) the Fv region of both the light and heavy chains of an
6 antibody; and
7 b) an effector molecule; wherein said Fv region and said
8 effector molecule are recombinantly fused to form a single molecule that has the binding
9 specificity of a monoclonal antibody selected from the group consisting of B1, B3, and
10 B5.

1 31. A method of improving the binding affinity of antibodies that lack a
2 serine at position 95 of V_H, said method comprising replacing the amino acid at position
3 95 of V_H with a Serine.

1 32. The method of claim 31, wherein said antibody is a Le^Y binding
2 antibody.

1 33. The method of claim 31, wherein said amino acid at position 95 of
2 V_H is a Tyrosine.

1 34. A recombinant DNA molecule that encodes a single chain fusion
2 protein, said recombinant DNA molecule comprising:
3 a) a DNA sequence that encodes the Fv region of both the light and
4 heavy chains of an antibody wherein said Fv region is selected from the group consisting
5 of B3(Fv): V_L M4L, B3(Fv): V_L S7T and B3(Fv): V_L M4L S7T; and
6 b) a DNA sequence that encodes an effector molecule.

1 35. A recombinantly produced single chain fusion protein comprising:
2 a) the Fv region of both the light and heavy chains of an antibody
3 wherein said Fv region is selected from the group consisting of B3(Fv): V_L M4L,
4 B3(Fv): V_L S75 and B3(Fv): V_L M4L S7T; and
5 b) an effector molecule.

1 36. A recombinant DNA molecule that encodes a single chain antibody,
2 said recombinant DNA molecule comprising a DNA sequence that encodes the Fv region
3 of both the light and heavy chains of an antibody; wherein said DNA sequences are
4 recombinantly fused to form a single molecule and wherein said Fv region is selected
5 from the group consisting of B3(Fv): V_L M4L, B3(Fv): V_L S7T and B3(Fv): V_L M4L
6 S7T.

1 37. A recombinantly produced single chain antibody comprising an Fv
2 region of both a light and a heavy chain of an antibody where said Fv region is selected
3 from the group consisting of B3(Fv): V_L M4L, B3(Fv): V_L S75 and B3(Fv): V_L M4L
4 S7T.

1 38. A recombinant DNA molecule that encodes a single chain fusion
2 protein, said recombinant DNA molecule comprising:
3 a) a DNA sequence which encodes the Fv region of a heavy chain
4 of a first antibody;

5 b) a DNA sequence which encodes the Fv region of a light chain of
6 a second antibody; and
7 c) a DNA sequence which encodes an effector molecule;
8 wherein said first antibody and said second antibody are different antibodies; wherein
9 said DNA sequences are recombinantly fused to form a single molecule; and wherein
10 said fusion protein specifically binds a Lewis^x carbohydrate antigen.

1 39. The recombinant DNA molecule of claim 38, wherein said effector
2 molecule is a *Pseudomonas* exotoxin.

1 40. The recombinant DNA molecule of claim 39, wherein said effector
2 molecule is selected from the group consisting of PE38, PE40, PE38KDEL and
3 PE38REDL.

1 41. The recombinant DNA molecule of claim 38, wherein said first
2 antibody is selected from the group consisting of B1, B3, and B5.

1 42. The recombinant DNA molecule of claim 38, wherein said second
2 antibody is selected from the group consisting of B1, B3, and B5.

1 43. The recombinant DNA molecule of claim 42, wherein said DNA
2 molecule encodes the fusion protein B5V_H-B3V_L-PE38.

1 44. The recombinant DNA molecule of claim 42, wherein said DNA
2 molecule encodes the fusion protein B3V_H-B5V_L-PE38.

1 45. A recombinantly produced single chain fusion protein comprising:
2 a) the Fv region of a heavy chain of a first antibody;
3 b) the Fv region of a light chain of a second antibody; and
4 c) an effector molecule;
5 wherein said first antibody and said second antibody are different antibodies and wherein
6 the Fv regions and said effector molecule are recombinantly fused to form a single
7 molecule that specifically binds a Lewis^x carbohydrate antigen.

1 46. The fusion protein of claim 45, wherein said effector molecule is a
2 *Pseudomonas* exotoxin.

1 47. The fusion protein of claim 46, wherein said effector molecule is
2 selected from the group consisting of PE38, PE40, PE38KDEL, PE38REDL.

1 48. The fusion protein of claim 45, wherein said first antibody is
2 selected from the group consisting of B1, B3, and B5.

1 49. The fusion protein of claim 45, wherein said second antibody is
2 selected from the group consisting of B1, B3, and B5.

1 50. The fusion protein of claim 49, wherein said DNA molecule
2 encodes the fusion protein B5V_H-B3V_L-PE38.

1 51. The fusion protein of claim 49, wherein said DNA molecule
2 encodes the fusion protein B3V_H-B5V_L-PE38.

1 52. A recombinant DNA molecule that encodes a single chain antibody,
2 said recombinant DNA molecule comprising:

3 a) a DNA sequence that encodes the Fv region of a heavy
4 chain of a first antibody; and

5 b) a DNA sequence that encodes the Fv region of a light chain
6 of a second antibody;

7 wherein said first antibody and said second antibody are different antibodies; further
8 wherein said DNA sequences are recombinantly fused to form a single molecule; and
9 wherein said single chain antibody binds a Lewis^x carbohydrate antigen.

1 53. The recombinant DNA molecule of claim 52, wherein said first
2 antibody is selected from the group consisting of B1, B3, and B5.

1 54. The recombinant DNA molecule of claim 52, wherein said second
2 antibody is selected from the group consisting of B1, B3, and B5.

1 55. The recombinant DNA molecule of claim 54, wherein said DNA
2 molecule encodes the antibody Fv B5V_H-B3V_L.

1 56. The recombinant DNA molecule of claim 54, wherein said DNA
2 molecule encodes the antibody Fv B3V_H-B5V_L.

1 57. A single chain antibody comprising:
2 a) an Fv region of a heavy chain of a first antibody; and
3 b) an Fv region of a light chain of a second antibody;
4 wherein said first antibody and said second antibody are different antibodies and wherein
5 said heavy and light chain are recombinantly fused to form a single molecule which
6 specifically binds a Lewis^x carbohydrate antigen.

1 58. The antibody of claim 57, wherein said first antibody is selected
2 from the group consisting of B1, B3, and B5.

1 59. The antibody of claim 58, wherein said second antibody is selected
2 from the group consisting of B1, B3, and B5.

1 60. The antibody of claim 59, wherein said antibody is the Fv
2 B5V_H-B3V_L.

1 61. The antibody of claim 59, wherein said antibody is the Fv
2 B3V_H-B5V_L.

1 62. A pharmaceutical composition comprising a recombinantly
2 produced single chain fusion protein in a concentration sufficient to inhibit tumor growth,
3 together with a pharmaceutically acceptable carrier wherein said fusion protein
4 comprises:
5 a) the Fv region of a heavy chain of a first antibody;
6 b) the Fv region of a light chain of a second antibody; and
7 c) an effector molecule;

8 wherein said first antibody and said second antibody are different antibodies and wherein
9 both of said Fv regions and said effector molecule are recombinantly fused to form a
10 single molecule that specifically binds cells bearing a Lewis^Y-related carbohydrate
11 antigen.

1 63. A method of detecting the presence or absence of a cell bearing a
2 Lewis^Y carbohydrate antigen in a patient, said method comprising the steps of:
3 a) removing a tissue or fluid sample from said patient;
4 b) adding an antibody to said sample wherein said antibody
5 comprises:
6 i) the Fv region of a heavy chain of a first antibody;
7 and
8 ii) the Fv region of a light chain of a second antibody;
9 wherein said first antibody and said second antibody are
10 different antibodies and wherein said Fv regions are
11 recombinantly fused to form a single molecule that
12 specifically binds a Lewis^Y carbohydrate antigen; and
13 c) detecting for the presence or absence of a binding complex
14 between the antibody and the antigen.

1 64. A method of killing or inhibiting the growth of cells bearing a
2 Lewis^Y antigen in a patient, said method comprising administering to the patient a
3 pharmaceutical composition in an amount sufficient to kill or inhibit the growth of said
4 cells, said composition comprising:
5 a) the Fv region of a heavy chain of a first antibody;
6 b) the Fv region of a light chain of a second antibody; and
7 c) an effector molecule;
8 wherein said first antibody and said second antibody are different antibodies and wherein
9 said Fv regions and said effector molecule are recombinantly fused to form a single
10 molecule that specifically binds a Lewis^Y carbohydrate antigen.

1 65. A recombinant DNA molecule that encodes a single chain fusion
2 protein, said recombinant DNA molecule comprising:
3 i) a DNA sequence that encodes a humanized Fv region of
4 both the light and heavy chains of an antibody selected from the group consisting of B1,
5 B3 and B5; and
6 ii) a DNA sequence that encodes an effector molecule;
7 wherein said DNA sequences are recombinantly fused to form a single molecule.

1 66. The recombinant DNA molecule of claim 65, wherein said DNA
2 sequence encodes an Fv region comprising a humanized variable heavy chain having the
3 amino acid sequence designated HumB3V_H in Figure 11.

1 67. The recombinant DNA molecule of claim 65, wherein said DNA
2 sequence encodes an Fv region comprising a humanized variable light chain having the
3 amino acid sequence designated HumB3V_L in Figure 11.

1 68. The recombinant DNA molecule of claim 65, wherein said DNA
2 sequence encodes an Fv region comprising a humanized variable heavy chain having the
3 amino acid sequence designated HumB3V_H in Figure 11A and a humanized variable light
4 chain having the amino acid sequence designated HumB3V_L in Figure 11.

1 69. The recombinant DNA molecule of claim 65, wherein said DNA
2 sequence encodes an Fv region comprising a humanized variable heavy chain having the
3 amino acid sequence designated HumB3V_H in Figure 11A with the except that the
4 sequence has an arginine at a position in said variable heavy chain designated as 82b in
5 Figure 11.

1 70. The recombinant DNA molecule of claim 65, wherein said effector
2 molecule is a *Pseudomonas* exotoxin.

1 71. The recombinant DNA molecule of claim 70, wherein said effector
2 molecule is selected from the group consisting of PE38, PE40, PE38KDEL and
3 PE38REDL.

1 72. A single chain fusion protein comprising:
2 a) a humanized Fv region of both the light and heavy chains of
3 an antibody selected from the group consisting of B1, B3 and B5; and
4 b) an effector molecule;
5 wherein the humanized Fv region and the effector molecule are recombinantly fused to
6 form a single molecule.

1 73. The fusion protein of claim 72, wherein the Fv region of said
2 protein comprises a humanized variable heavy chain having the amino acid sequence
3 designated HumB3V_H in Figure 11.

1 74. The fusion protein of claim 72, wherein the Fv region of said
2 protein comprises a humanized variable heavy chain having the amino acid sequence
3 designated HumB3V_L in Figure 11.

1 75. The fusion protein of claim 72, wherein the Fv region of said
2 protein comprises a humanized variable heavy chain having the amino acid sequence
3 designated HumB3V_H in Figure 11 and a humanized variable light chain having the
4 amino acid sequence designated HumB3V_L in Figure 11.

1 76. The fusion protein of claim 72, wherein the Fv region of said
2 protein comprises a humanized variable heavy chain having the amino acid sequence
3 designated HumB3V_H in Figure 11 with the exception that the sequence has an arginine
4 at a position in said variable heavy chain designated as 82b in Figure 11 replaced serine
5 with arginine at the position in the variable heavy chain designated as 82b in Figure 11.

1 77. The fusion protein of claim 72, wherein said effector molecule is a
2 *Pseudomonas* exotoxin.

1 78. The fusion protein of claim 77, wherein said *Pseudomonas* exotoxin
2 is PE38, PE40, PE38KDEL and PE38REDL.

1 79. A recombinant DNA molecule that encodes a humanized Fv region
2 of both the light and heavy chains of an antibody wherein said antibody is a monoclonal
3 antibody selected from the group consisting of B1, B3 and B5.

1 80. The recombinant DNA molecule of claim 79, wherein said DNA
2 sequence encodes an Fv region comprising a humanized variable heavy chain having the
3 amino acid sequence designated HumB3V_H in Figure 11.

1 81. The recombinant DNA molecule of claim 79, wherein said DNA
2 sequence encodes an Fv region comprising a humanized variable light chain having the
3 amino acid sequence designated HumB3V_L in Figure 11.

1 82. The recombinant DNA molecule of claim 79, wherein said DNA
2 sequence encodes an Fv region comprising a humanized variable heavy chain having the
3 amino acid sequence designated HumB3V_H in Figure 11a and a humanized variable light
4 chain having the amino acid sequence designated HumB3V_L in Figure 11.

1 83. The recombinant DNA molecule of claim 79, wherein said DNA
2 sequence encodes an Fv region comprising a humanized variable heavy chain having the
3 amino acid sequence designated HumB3V_H in Figure 11A with the exception that there is
4 an arginine at a position in said variable heavy chain designated as 82b in Figure 11.

1 84. A recombinantly produced protein comprising a humanized Fv
2 region of both a light and a heavy chain of an antibody wherein said antibody is a
3 monoclonal antibody selected from the group consisting of B1, B3 and B5.

1 85. The protein of claim 84, wherein said protein comprises a
2 humanized variable heavy chain having the amino acid sequence designated HumB3V_H in
3 Figure 11.

1 86. The protein of claim 84, wherein said protein comprises a
2 humanized variable heavy chain having the amino acid sequence designated HumB3V_L in
3 Figure 11.

1 87. The protein of claim 84, wherein said protein comprises a
2 humanized variable heavy chain having the amino acid sequence designated HumB3V_H in
3 Figure 11 and a humanized variable light chain having the amino acid sequence
4 designated HumB3V_L in Figure 11.

1 88. The protein of claim 84, wherein said protein comprises a
2 humanized variable heavy chain having the amino acid sequence designated HumB3V_H in
3 Figure 11 with the exception that there is a variable heavy chain is back arginine at a
4 position in the variable heavy chain designated as 82b in Figure 11.

1 89. A pharmaceutical composition comprising a recombinantly
2 produced single chain fusion protein in a concentration sufficient to inhibit tumor growth,
3 together with a pharmaceutically acceptable carrier wherein said fusion protein
4 comprises:

5 a) a humanized Fv region of both a light and a heavy chain of an
6 antibody wherein said antibody is a monoclonal antibody selected from the group
7 consisting of B1, B3 and B5; and

8 b) an effector molecule;
9 wherein both of said Fv regions and said effector molecule are recombinantly fused to
10 form a single molecule that has the binding specificity of a monoclonal antibody selected
11 from the group consisting of B1, B3 and B5.

1 90. The composition of claim 89, wherein said effector molecule is a
2 *Pseudomonas* exotoxin.

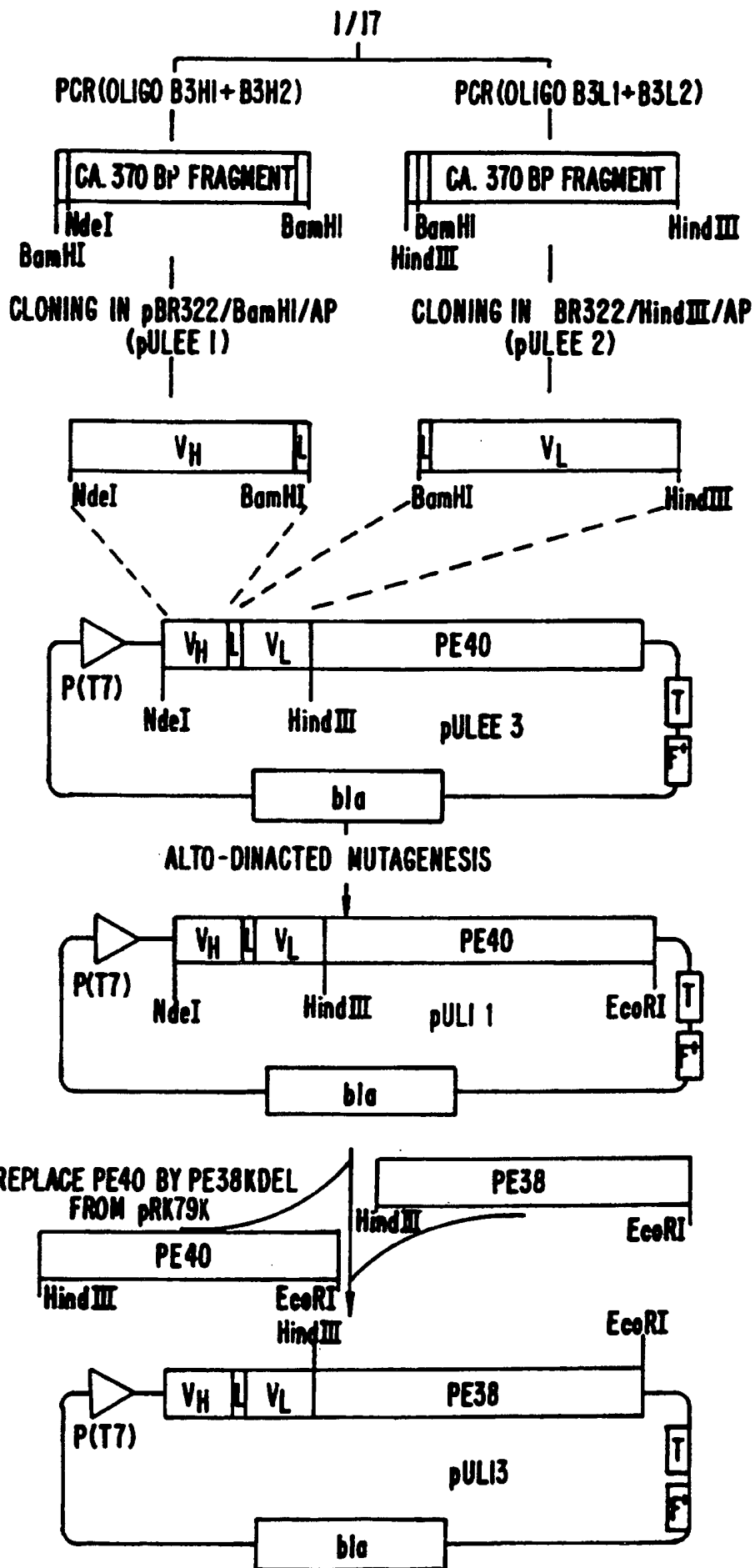
1 91. The composition of claim 90, wherein said effector molecule is
2 selected from the group consisting of PE38, PE40, PE38KDEL, PE38REDL.

1 92. The composition of claim 89, wherein said humanized Fv region is
2 a humanized B3(Fv) region.

1 93. A method of detecting the presence or absence of a cell bearing a
2 Lewis^Y carbohydrate antigen in a patient, said method comprising the steps of:

- 3 a) removing a tissue or fluid sample from said patient;
4 b) adding an antibody to said sample wherein said antibody
5 comprises:
6 i) a humanized Fv region of both a light and a heavy chain
7 of an antibody wherein said antibody is a monoclonal antibody selected from the group
8 consisting of B1, B3 and B5; and
9 ii) an effector molecule;
10 further wherein said Fv regions are recombinantly fused to
11 form a single molecule that has the binding specificity of a monoclonal antibody selected
12 from the group consisting of B1, B3 and B5; and
13 c) detecting for the presence or absence of a binding complex
14 between the antibody and the antigen.

- 1 94. A method of killing or inhibiting the growth of cells bearing a
2 Lewis^y antigen in a patient, said method comprising administering to the patient a
3 pharmaceutical composition in an amount sufficient to kill or inhibit the growth of said
4 cells, said composition comprising:
5 a) a humanized Fv region of both a light and a heavy chain of an
6 antibody wherein said antibody is a monoclonal antibody selected from the group
7 consisting of B1, B3 and B5; and
8 b) an effector molecule;
9 wherein both of said Fv regions and said effector molecule are recombinantly fused to
10 form a single molecule that has the binding specificity of a monoclonal antibody selected
11 from the group consisting of B1, B3 and B5.

**FIG 1A.**

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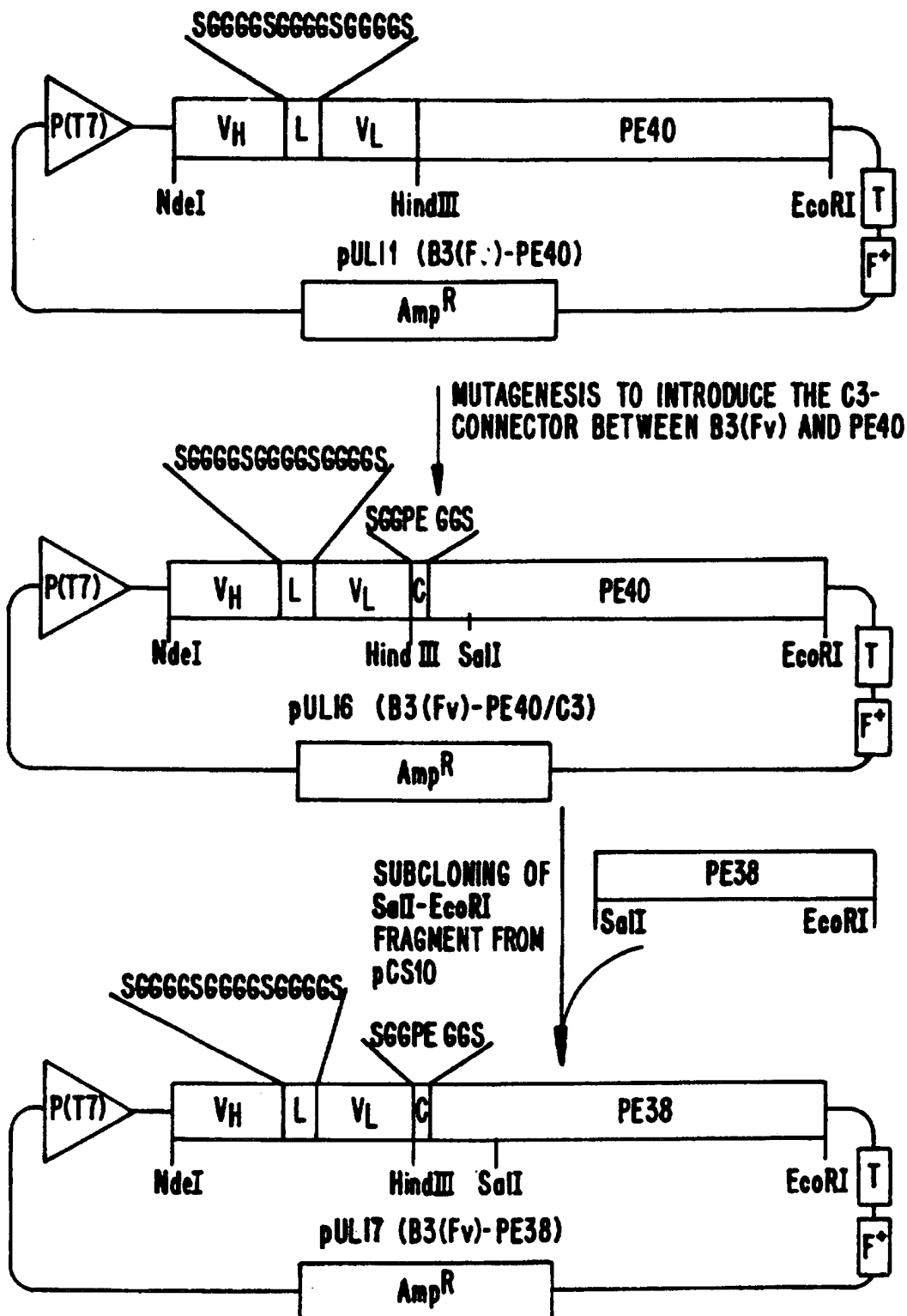
**FIG. 1B.**

FIG. 2A-1.

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601 CCAACCGATTTTCTGGGGTCCCAGACAGGTTTCAGTGGCAGTGGATCAGGG 650
N R F S G V P D R F S G S G S G
651 ACAGATTTCACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGT 700
T D F T L K I S R V E A E D L G V
701 TTATTACTGCTTTCAAGGTTACATGTTCCATTACGTTCTGGCTCGGGGA 750
Y Y C F Q G S N V P F T F G S G T

HindIII

751 CAAAGCTGGAAATTAAAGCTTT..... 772
K L E I K A F -> PE40

FIG. 2A-2.

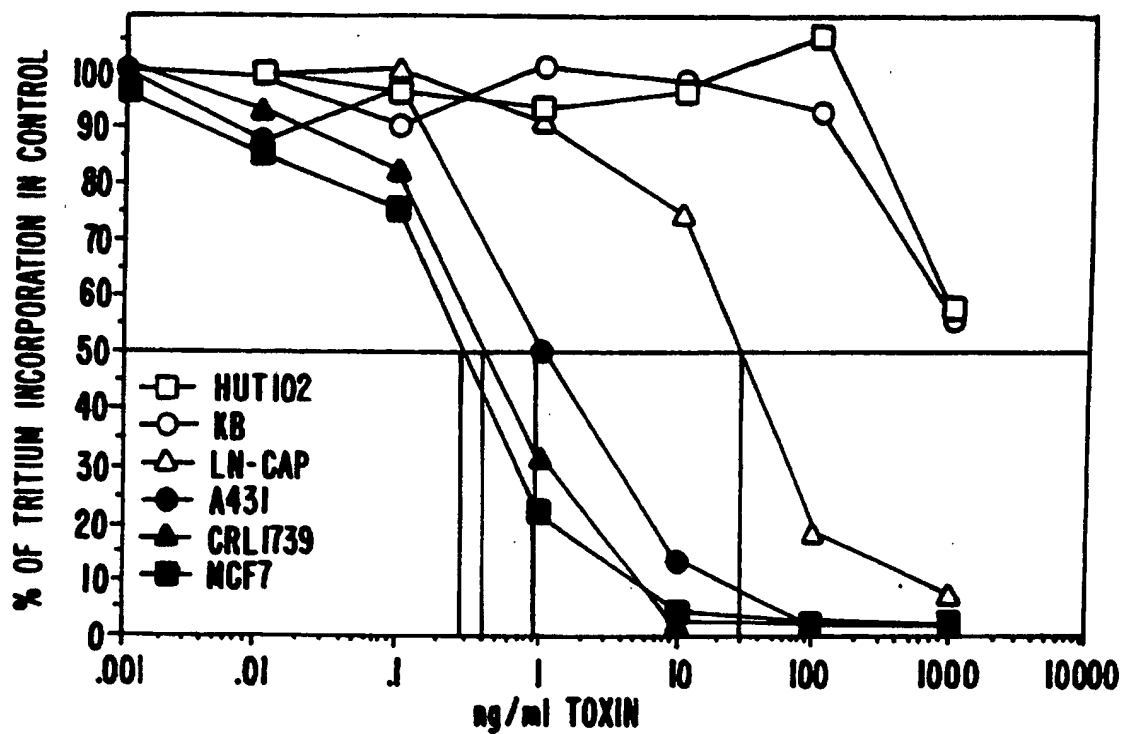
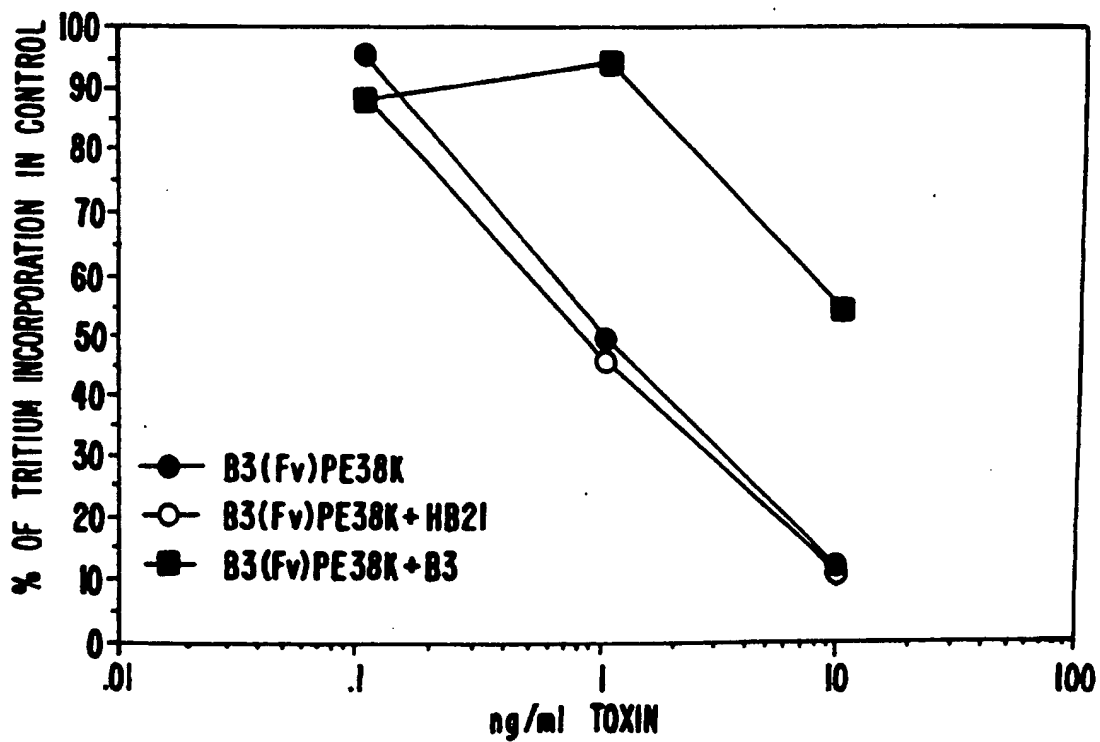
721 CACATGTTCCATTACGTTCTGGCTCGGGGACAAAGCTGGAAATTAAATAA 770
H V P F T F G S G T K L E I K *

EcoRI

771 TGAATTCC.. 779
* -> TERM

FIG. 2B.

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**FIG. 3A.****FIG. 3B.**

Cytotoxicity towards A431 cells was measured by the inhibition of incorporation of ³H-leucine into cell protein, following 2 hours (open symbols) or 20 hours (solid symbols) of incubation of the cells with serial dilutions of immunotoxins in PBS + 0.2% BSA.

5 Figure 5 shows antigen binding of B1(Fv)-PE38, B3(Fv)-PE38 and B5(Fv)-PE38. Antigen binding was estimated by competition of [¹²⁵I]-B1 IgG (A) or [¹²⁵I]-B3 IgG (B) binding to A431 cells at 4°C.

Figure 6 shows stability data for B1(Fv)-PE38, B3(Fv)-PE38, and B5(Fv)-PE38. The immunotoxins were diluted in PBS to 0.1 mg/ml and incubated at 10 37°C for 4 hours. (A) The molecular forms of the immunotoxins were then analyzed by size exclusion chromatography at 4°C. The monomer peak elutes at 18-20 ml, while the aggregates elute at 11-13 ml. Chromatograms of the proteins prior to incubation at 37°C are shown by broken lines. The proteins after the incubation at 37°C are shown by solid lines. (B) Cytotoxic activity of immunotoxins before (open symbols) or after (solid 15 symbols) incubation at 37°C. Other details are as in Figure 5(B).

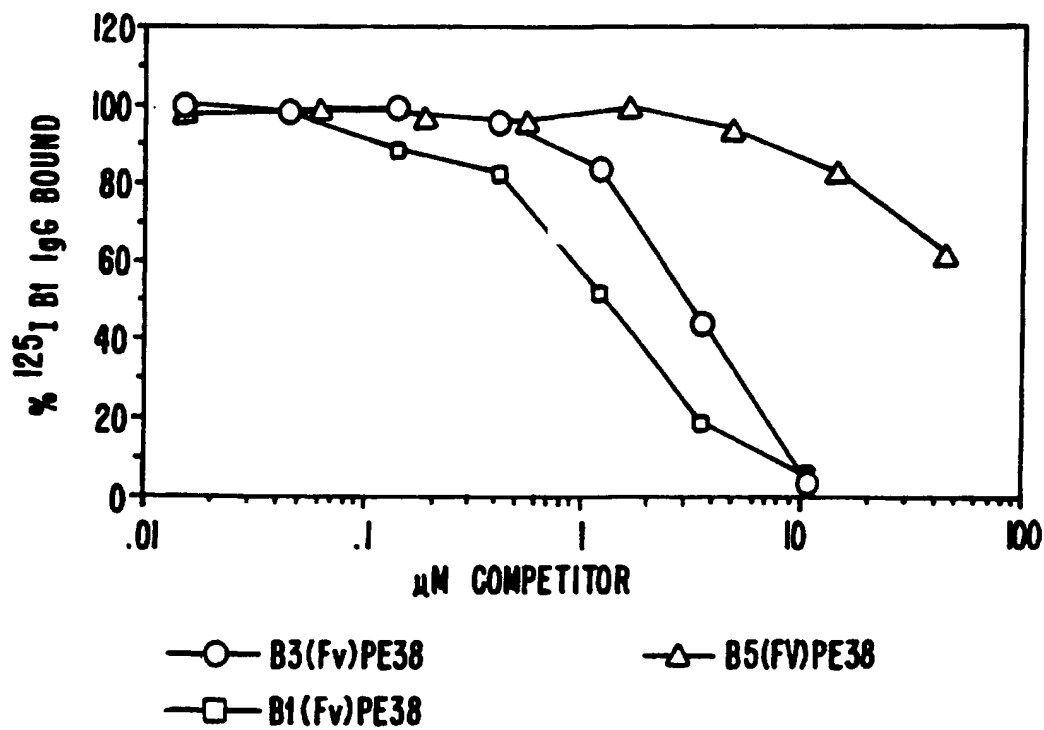
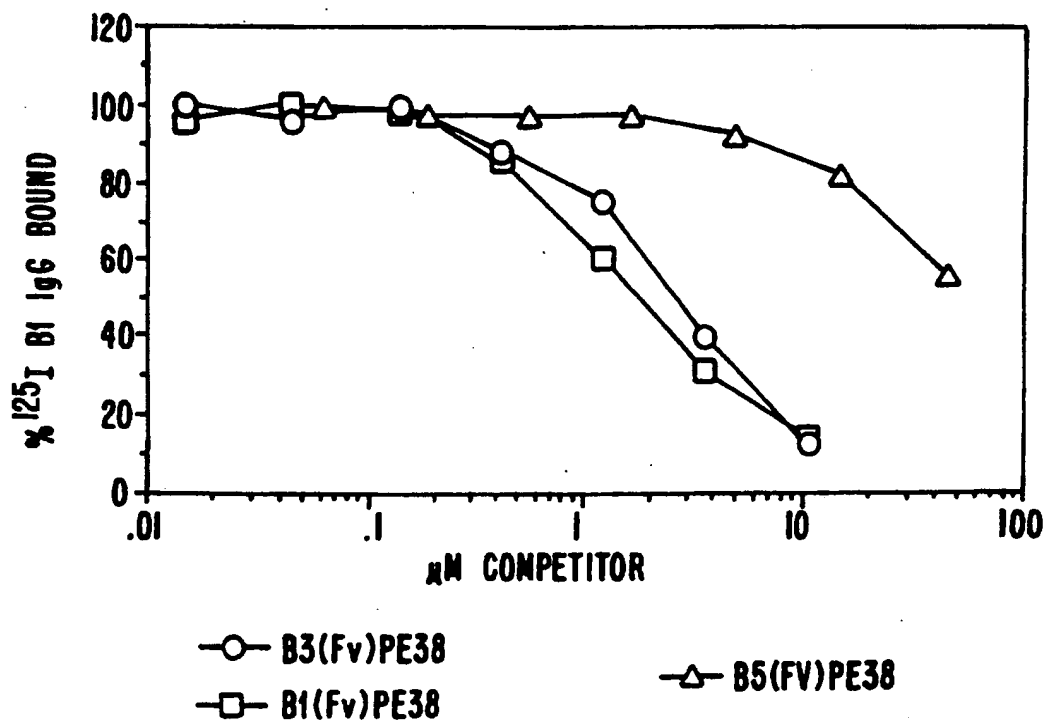
Figure 7 shows blood levels of B3(Fv)-PE38KDEL in mice. Balb/c mice were injected intravenously with 10 µg of B3(Fv)-PE38KDEL and immunotoxin levels were measured at different time periods. Bars indicate the standard deviation.

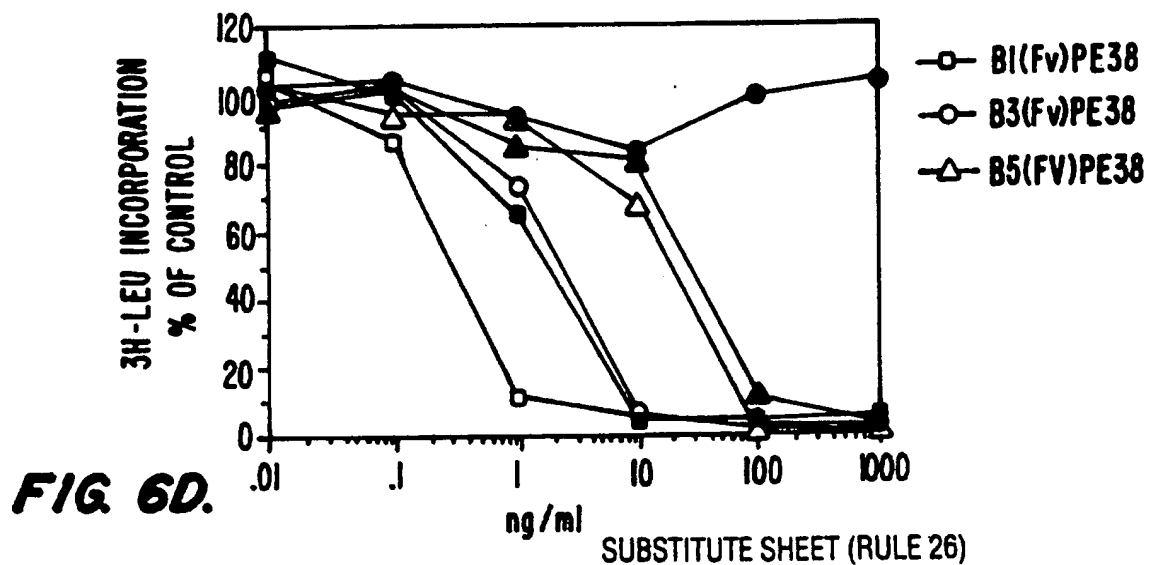
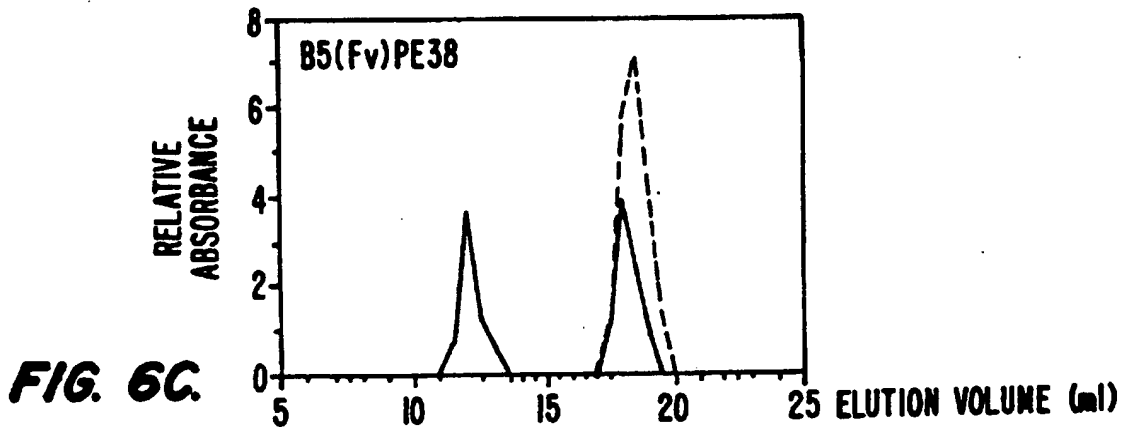
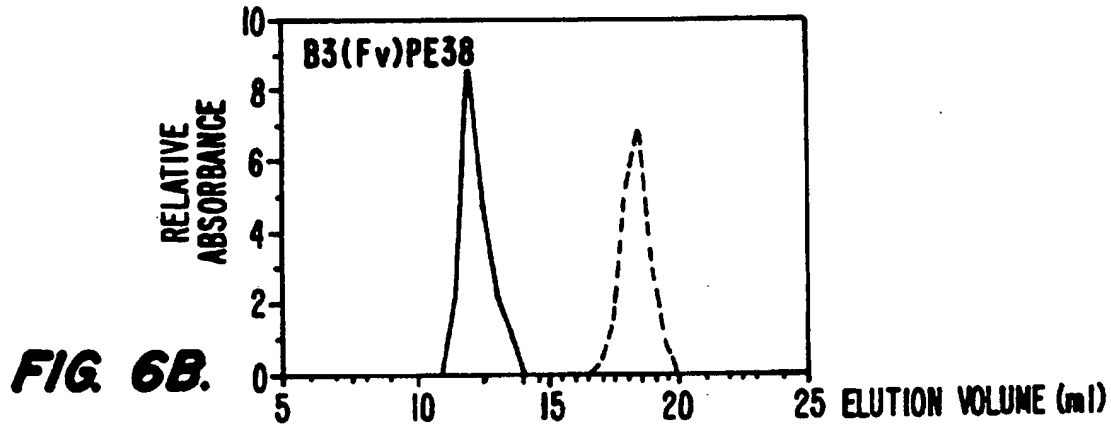
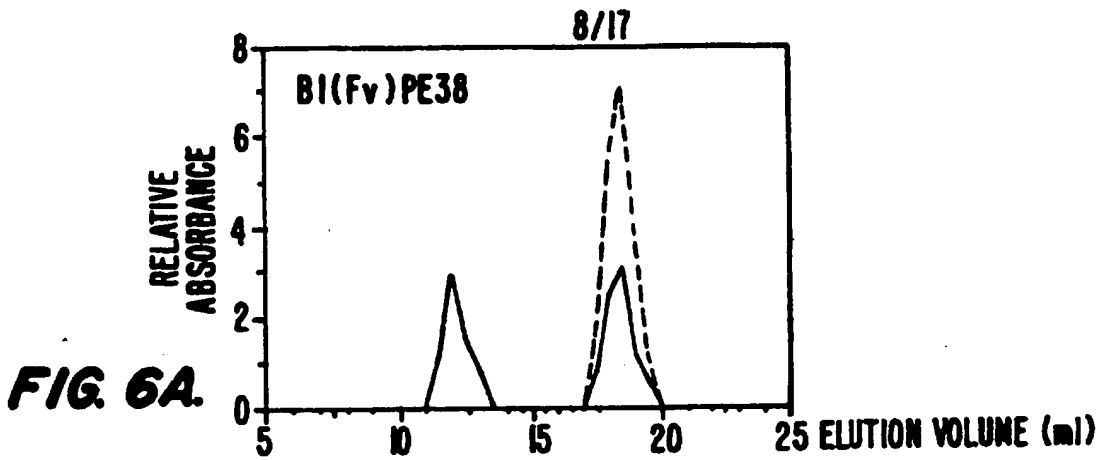
Figure 8 illustrates the effect of B3(Fv)-PE38KDEL on the growth of 20 A431 tumors in nude mice. Mice were injected with 3 x 10⁶ A431 cells on day 0 and treated beginning on day 4 with intravenous injections every 12 hrs x 6. A: (○) untreated; (●) 10 µg B3(Fv)-PE38KDEL; B: (□) 2.5 µg B3; (■) 5 µg B3(Fv)-PE38KDEL; C: (Δ) 2.5 µg anti-Tac (Fv)PE38KDEL; (▲) 2.5 µg B3(Fv)-PE38KDEL; (—○—) 0.5 µg B3(Fv)-PE38KDEL; D: treatment began on day 7 25 with intravenous injections every 12 hrs x 8. (○) untreated, (■) 5 µg B3(Fv)-PE38KDEL. Bars = 1 standard deviation.

Figure 9 illustrates the construction of plasmids for expression of B3-B5 chimeric Fv single chain immunotoxins. L indicates the (Gly₄Ser)₃ linker which connects the V_H to the V_L in the single-chain Fv configuration.

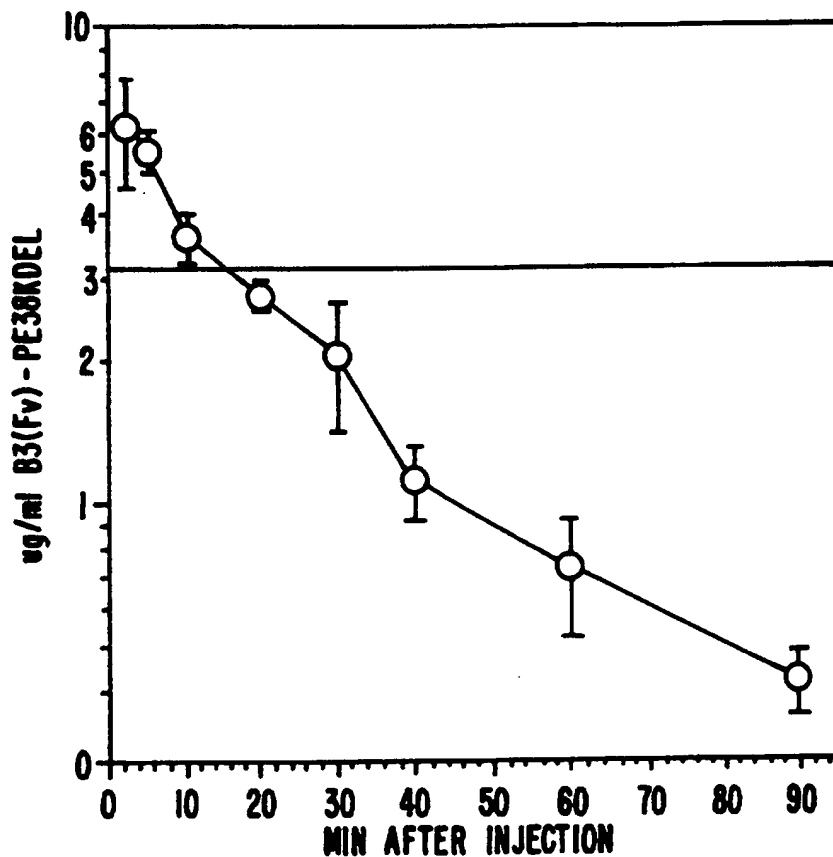
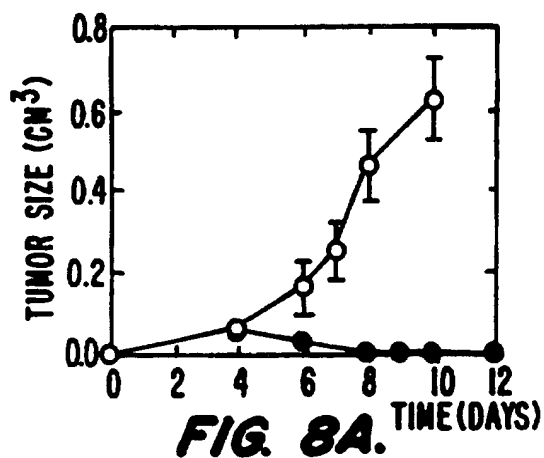
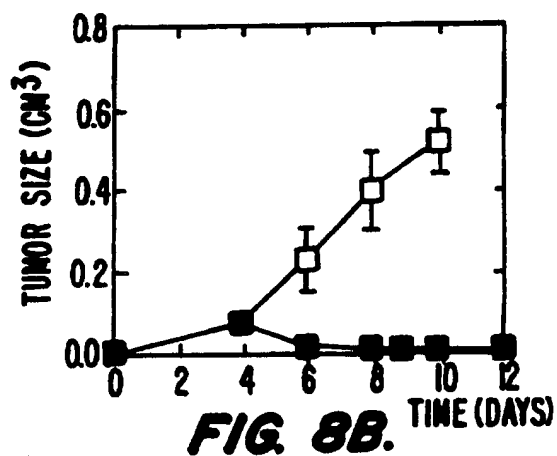
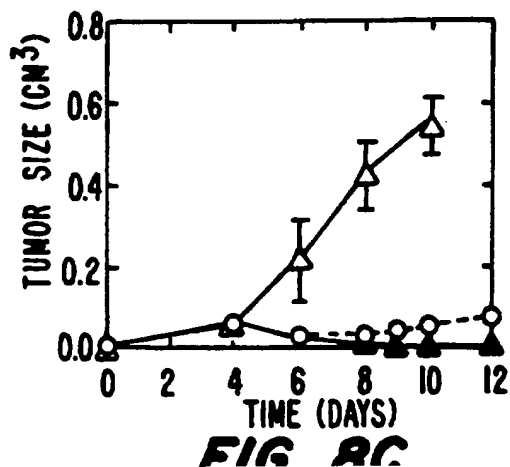
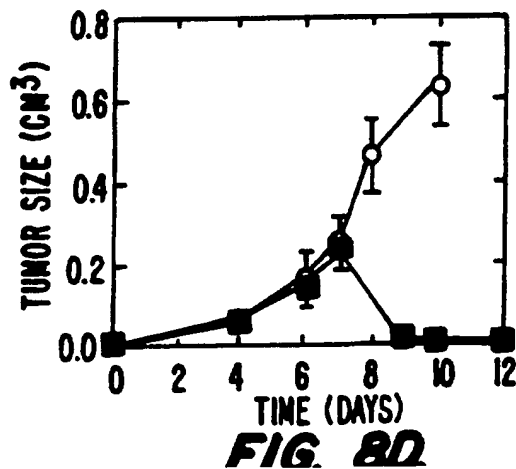
30 Figure 10 shows the cytotoxic activity of immunotoxins B3(Fv)-PE38, B3V_H-B5V_L-PE38 and B3(Fv)-PE38: V_L M4L S7T following incubation in PBS at 37°C. A431 epidermoid carcinoma cells were incubated with aliquots of the immunotoxins

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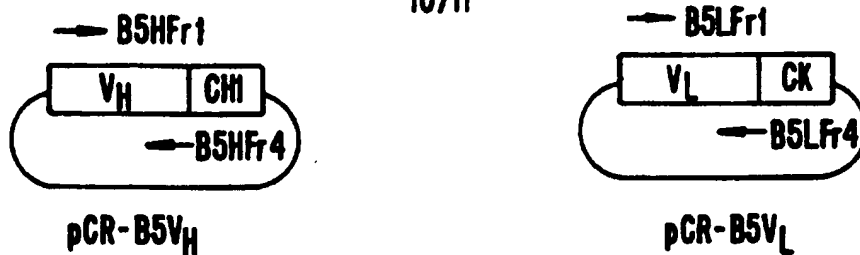
**FIG. 5A.****FIG. 5B.**



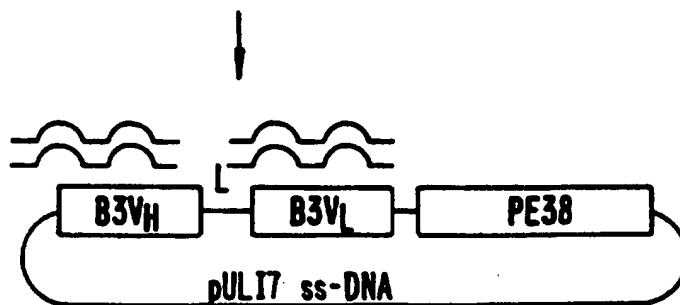
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**FIG. 7.****FIG. 8A.****FIG. 8B.****FIG. 8C.****FIG. 8D.**

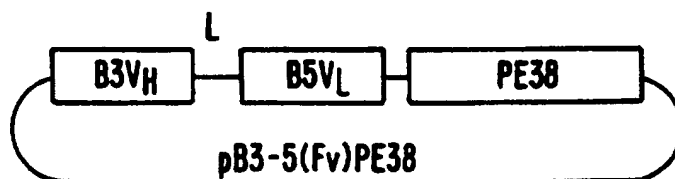
10/17



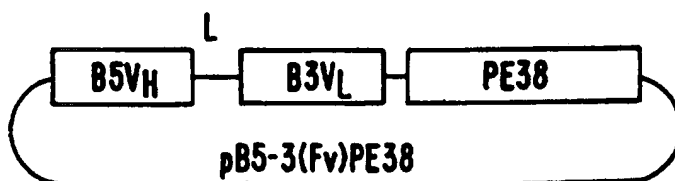
PCR AMPLIFY VARIABLE
SEGMENTS, ANNEAL PCR
PRODUCTS SEPARATELY OR
COMBINED TO THE TEMPLATE



EXTEND AND LIGATE
TRANSFORM *E. coli* DH5α



OR



OR

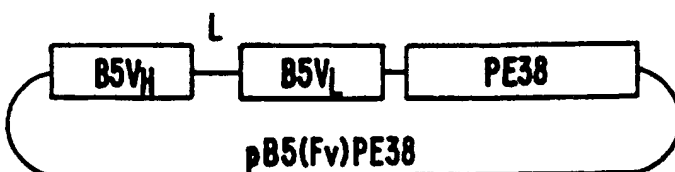


FIG. 9.
SUBSTITUTE SHEET (RULE 26)

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FIG. 10A.

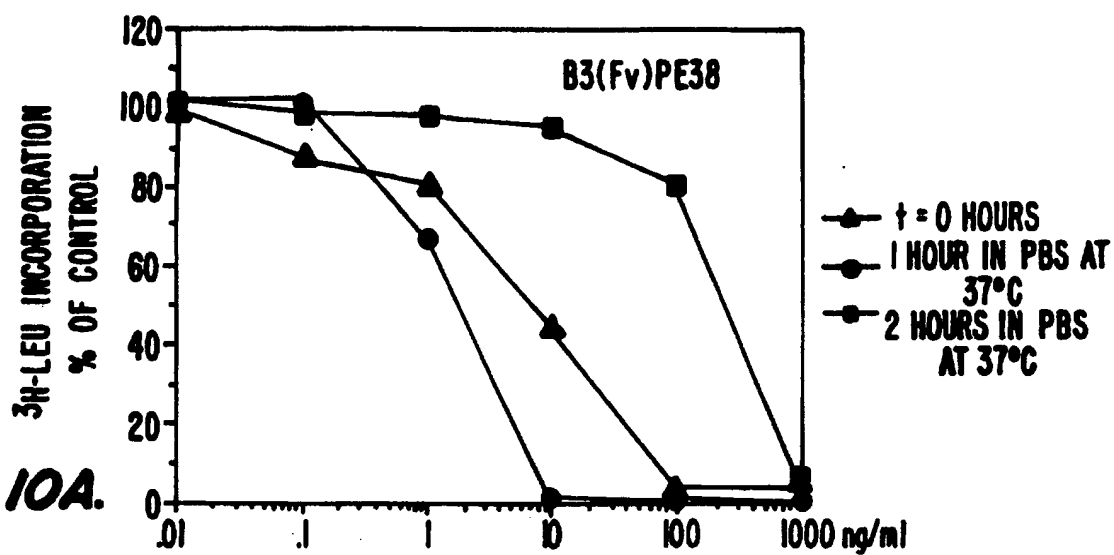


FIG. 10B.

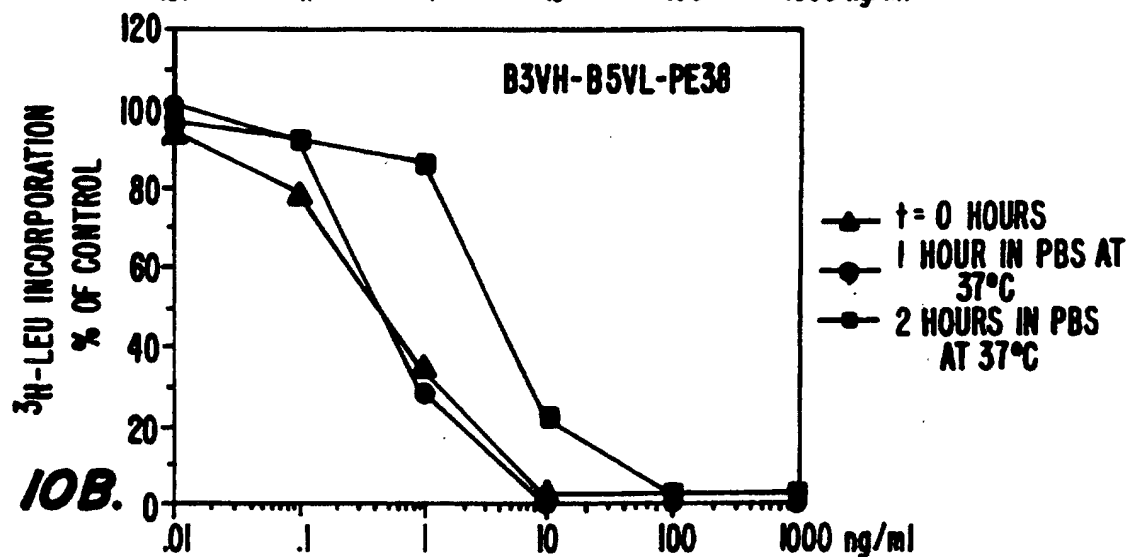
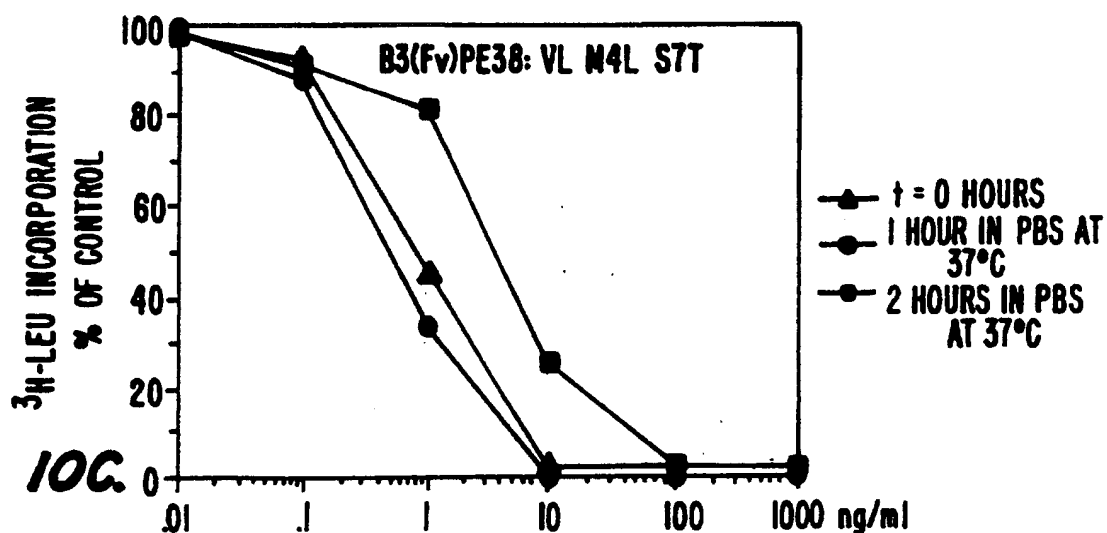


FIG. 10C.



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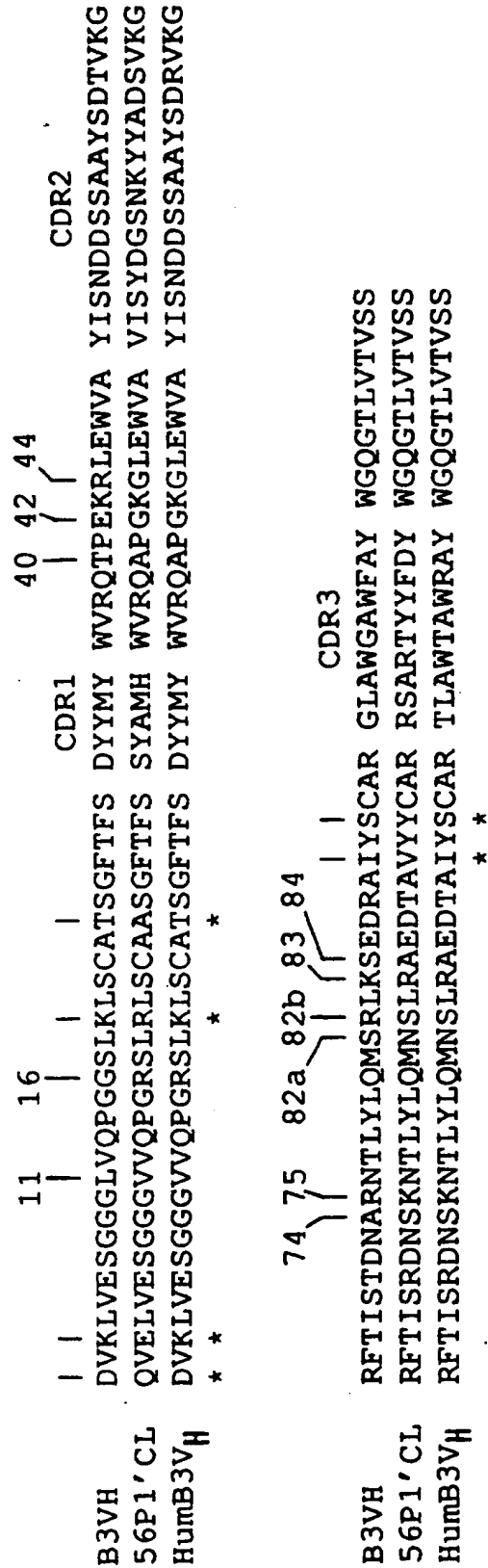


FIG. 11A.

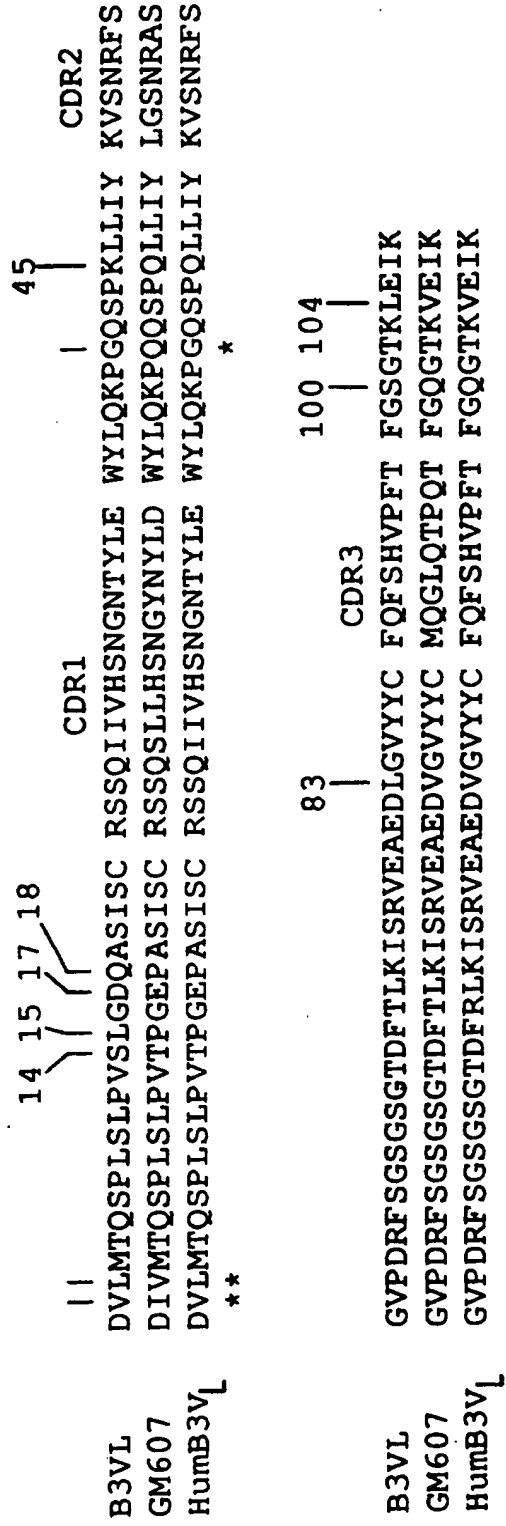
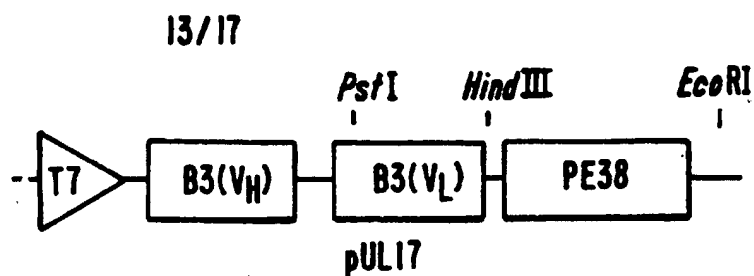
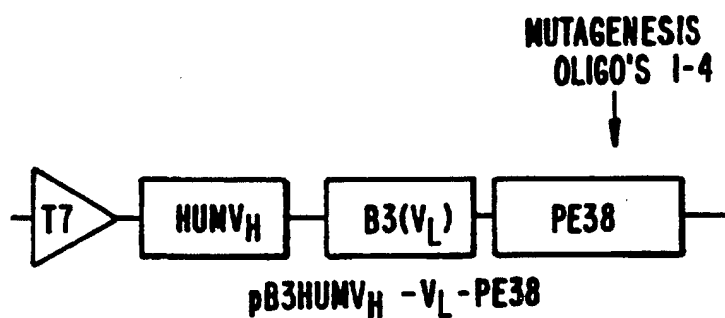
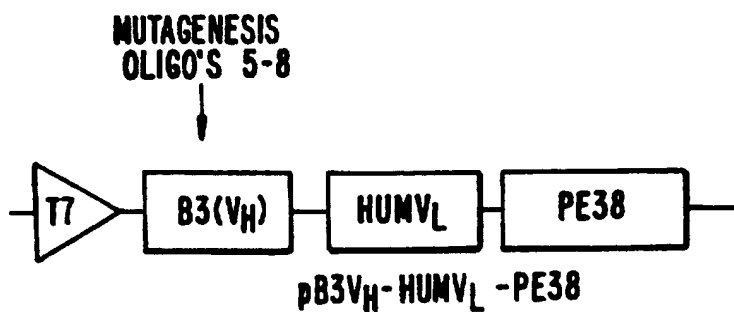
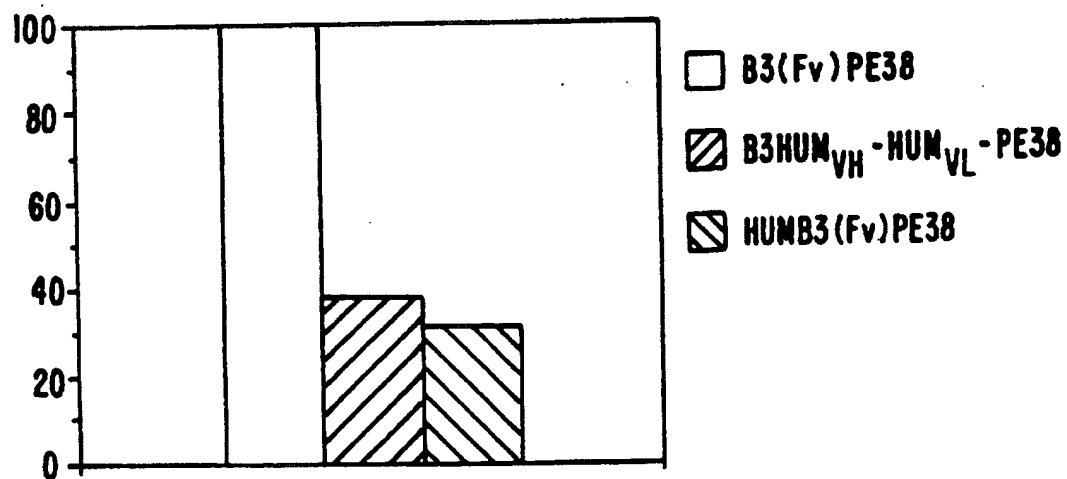
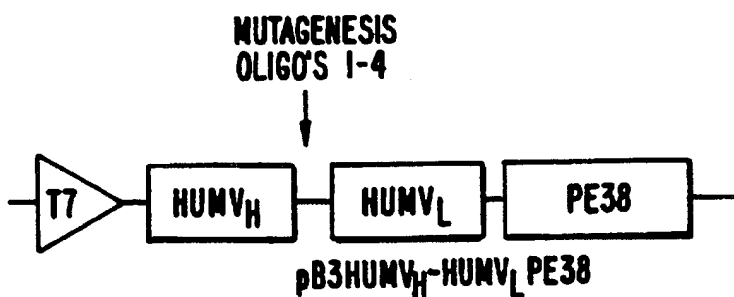


FIG. 11B.

FIG. 12A.**FIG. 12B.****FIG. 12C.****FIG. 12D.****FIG. 14.**

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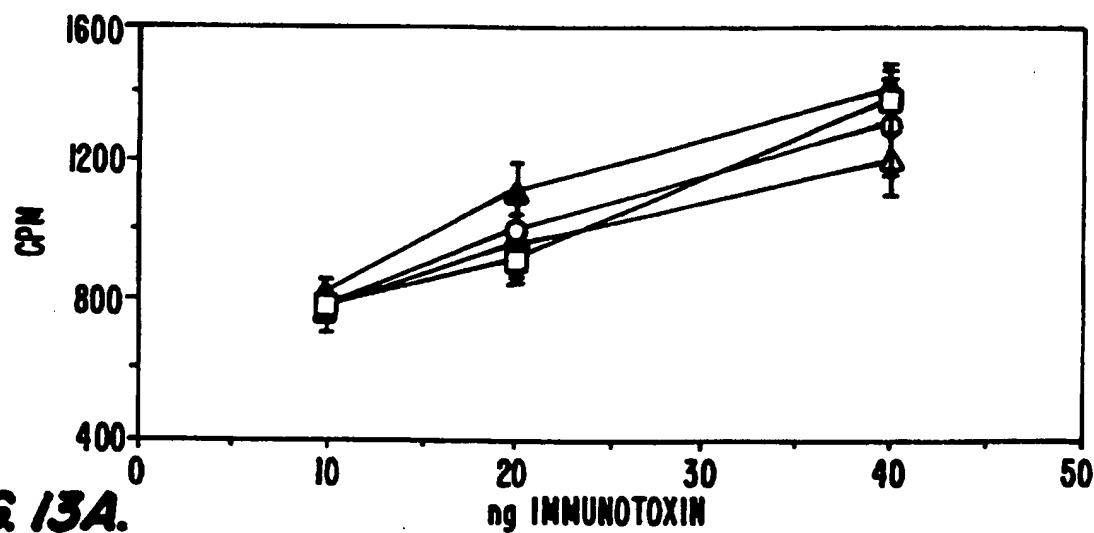


FIG. 13A.

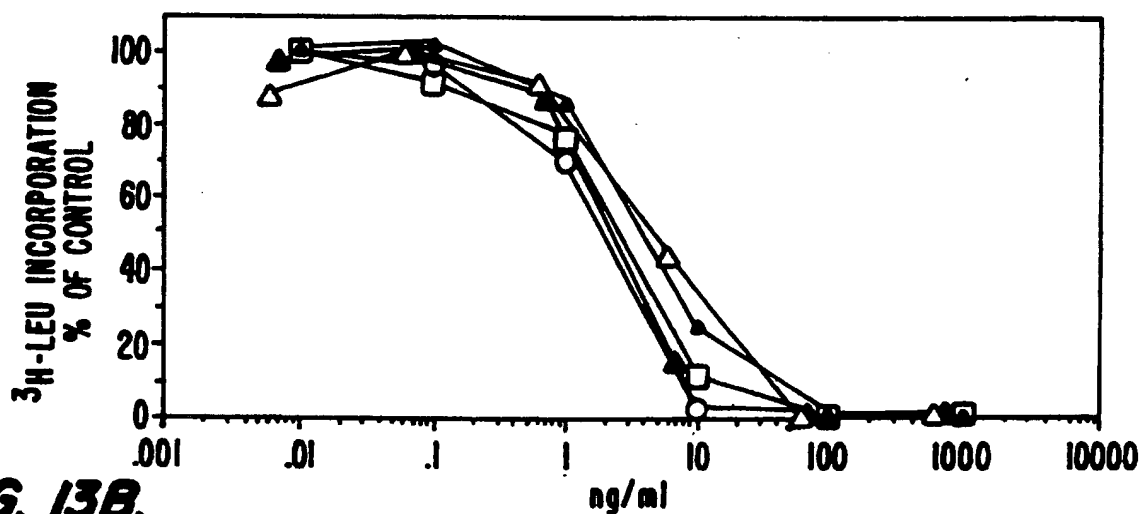


FIG. 13B.

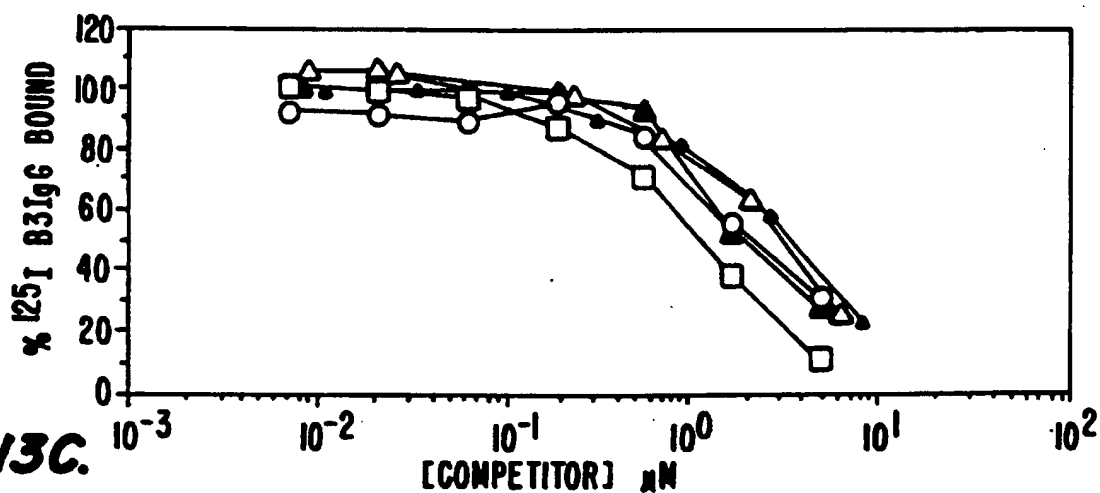


FIG. 13C.



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GAGGTGCAGCTGGTGGAAATCTGGAGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAA
 E V Q L V E S G G G L V K P G G S L K
 E V Q L V E S G G G L V K P G G S L
 CTCTCCTGTGCAGCCTCTGGATTCATTTTCAGTGACAATTACATGTATTGGGTTCGC
 L S C A A S G F I F S D N Y M Y W V R
 CDR 1

CAGACTCCGGAGAAGAGGCTGGAGTGGGTCGCAACCATTAGTGATGGTGGCACTTAT
 Q T P E K R L E W V A T I S D G G T Y
 CDR 2

ATCGACTATTCAGACAGTGTGAAGGGGCGATTACCATCTCCAGAGACAATGCCAAG
I D Y S D S V K G R F T I S R D N A K

AATAATCTGTACTTGCAAATGAGCAGTCTGAGGTCTGAGGACACAGGCATGTATTAT
 N N L Y L Q M S S L R S E D T G M Y Y

TGTGGAAGGAGTCCGATCTACTATGATTACGCCCCGTTTACTTACTGGGGCCAAGGG
 C G R S P I Y Y D Y A P F T Y W G Q G
 CDR 3

ACTCTGGTCACTGTCTCTGCAGCCAAAACGACACCCCCATCTGTCTATCCACTGGCC
 C L V T V S A A K T T P P S V Y P L A

CCTGGATCTGCT
~~P G S A~~

FIG. 15A.

GATGTTGTGATGACCCAGACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCC
 D V V M T Q T P L S L P V S L G D Q A
 D V V M T Q T P L S L P V S L G D
 TCCATCTCTTGCAGATCTAGTCAAAACCTTGTACACAGTGATGGAAAAACCTATTTA
 S I S C R S S O N L V H S D G K T Y L
 CDR 1

CATTGGTTCCTGCAGAAGCCTGGCCAGTCTCCAACGCTCCTGATCTACAAAGTTTCC
H W F L Q K P G Q S P T L L I Y K V S
 CDR 2

AACCGATTTTCTGGGGTCCCAGACAGGTTTCAGTGGCAGTGGATCAGGGACAGATTTC
N R F S G V P D R F S G S G S G T D F

ATACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAA
 I L K I S R V E A E D L G V Y F C S O

AGTACACATGTTCCGCTCACGTTCCGTGCTGGGACCAAGCTGGAGCTGAAACGGGCT
S T H V P L T F G A G T K L E L K R A
 CDR 3

GATGCTGCACCAACTGTATCCATCTTCCCACCA
~~D A A P T V S I F P P~~

FIG. 15B.

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GAGGTGAAGCTGGTGGGAATCTGGAGGAGGCTTAGTGCAGCCTGGAGGGTCCCTGAAA
 E V K L V E S G G G L V Q P G G S L K
 E V K L V E S G G G L V Q P G
 CTCTCCTGTGCAACCTCTGGATTTACTTTCACTGACTATTACATGTATTGGGTTTCGC
 L S C A T S G F T F S D Y Y M Y W V R
 CDR 1
 CAGACTCCAGAGAAGAGGCTGGAGTGGGTCGCATACATTAGTAATGGTGGTGGTAGC
 Q T P E K R L E W V A Y I S N G G S
 CDR 2
 ACCTATTATCCAGACACTGTAAAGGGCCGATTCACCATCTCCAGAGACAACGCCAAG
T Y Y P D T V K G R F T I D R D N A K
 AACACCCTGTACCTGCAGATGAGCCGTCTGAAGTCTGAGGACACAGCCATGTATTAC
 N T L Y L Q M S R L K S E D T A M Y Y
 TGTGCAAGGGGGCTCTCTGATGGTTCCTGGTTTGCTTACTGGGGCCAAGGGACTCTG
 C A R G L S D G S W F A Y W G Q G T L
 CDR 3
 GTCACTGTCTCCTCAGGCGGAGGCGGATCCGGT
 V T V S S ~~G G G G S G~~

FIG. 16A.

GATGTTTTGTTGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCC
 D V L L T Q T P L S L P V S L G D Q A
 D V L L T Q T P L S L P V S L
 TCTATTTCTTGTAGATCTAGTCAGAGCATTGTACATAGTAATGGAAACACCTATTTA
 S I S C R S S O S I V H S N G N T Y L
 CDR 1
 GAATGGTACCTGCAGAAACCAGGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCC
E W Y L Q K P G Q S P K L L I Y K V S
 CDR 2
 AACCGATTTTCTGGGGTCCCAGACAGGTTCACTGGCAGTGGATCAGGGACAGATTTC
N R F S G V P D R F S G S G S G T D F
 ACACTCAAGATCAGCAGAGTGGAGGCTFAGGATCTGGGAGTTTATTACTGCTTTCAA
 T L K I S R V E A E D L G V Y Y C F O
 GGTTACATGTTCCATTACGTTTCGGCTCGGGGACAAAGTTGGAAATAAAGCGGGCT
G S H V P F T F G S G T K L E I K R A
 CDR 3
 GATGCTGCACCAACTGTATCCATCTTCCCACCA
~~D A A P T V S I F P P~~

FIG. 16B.

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VH:
 DVKLVEGGGLVQPGGSLKLSCATSGFTS (DYMY) WVRQTPKRLWVA (YISNDDSSAAYSDTVKG)
 RFTISTDNARNTLYLQMSRLKSEDTAIYSCAR (GLAWGAWFAY) WQGGTLVTVSS

LINKER:
 GGGGGGGGGGGGS

VL:
 DVLMTQSPVSLPVSIGDQASISC (RSSQIIVHSNGNTYLE) WYLQKPGQSPKLLIY (KVSNRFS)
 GVPDRFSFSFTDFTLKISRVEAEDLGVYYC (FQGSHPVFT) FGSGTKLEIK

FIG. 17.

VH:
 GATGTGAAGCTGGTGGAGTCTGGGGGAGGCGTCGTGCAGCCCGGCGCTCCCTGAAACTCTCCTGTGCAACCTCTG
 GATTCACCTTTCAGTGACTATTACATGTATTGGGTTCCCGAGGCCCGGGCAAGGGCTGGAGTGGTCCGCATACAT
 TAGTAATGATGATAGTTCCGCCGCTTATTCAGACACTGTAAAGGGCCGTTACCATCTCTAGAGACAAAGCAAG
 AACACCCCTACCTGCAAAATGAACCGTCTGCGCGGAGGACACAGCCATATATTCTGTGCAAGAGGACTGGCCT
 GGGGAGCCCTGGTTTGCTTACTGGGGCCCAAGGACTCTGGTCACTGTCCTCTCA

LINKER: ggcggaggcgatccggtggtggcgatctggaggtggcggaagc

VL:
 GATGTGCTGATGACCCAGTCTCCATTGAGTTTACCTGTCAACCCCGGAGAGCCGGCTCCATCTCTGCAGATCTA
 GTCAGATCATTTGTACATAGTAATGGAACACCTATTAGAAATGGTACCTGCAGAAACAGGCCAGTCTCCACAGCT
 GCTGATCTACAAAGTTTCCAAACCGATTTTCTGGGTCCCAGACAGGTTTCAGTGGCAGTGGATCAGGGACAGATTTC
 ACACTCAAGATCAGCAGAGTGGAGGCTGAGGACGTCGGAGTTTATTACTGCTTCAAGGTTACATGTTCCATTCA
 CGTTCGGCCAGGGTACCAAGGTCGAAATTAA

FIG. 18.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/62 C12N15/13 C07K19/00 C07K16/30 A61K47/42
 A61K39/395 G01N33/577 G01N33/574 C07K16/00 C07K16/46
 //C07K14/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 07286 (THE UNITED STATES OF AMERICA) 15 April 1993 cited in the application see the whole document	1-30
X	BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1198, 1994 AMSTERDAM, THE NETHERLANDS, pages 27-45, XP 000564482 U. BRINKMANN ET AL. 'Immunotoxins against cancer' cited in the application see the whole document	1-30

-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *Z* document member of the same patent family

Date of the actual completion of the international search

28 February 1996

Date of mailing of the international search report

25 MARCH 1996

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
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 Fax: (+ 31-70) 340-3016

Authorized officer

Nooij, F

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 88, no. 19, 1 October 1991 WASHINGTON, DC, USA, pages 8616-8620, U. BRINKMANN ET AL. 'B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice.' cited in the application see abstract see figures 1,2</p> <p>---</p>	1-30
X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 89, no. 7, 1 April 1992 WASHINGTON, DC, USA, pages 3075-3079, XP 000264181 U. BRINKMANN ET AL. 'Independent domain folding of Pseudomonas exotoxin and single-chain immunotoxins: Influence of interdomain connections.' cited in the application see figure 1</p> <p>---</p>	1-22
X	<p>BIOCONJUGATE CHEMISTRY, vol. 5, no. 4, July 1994 WASHINGTON, DC, USA, pages 321-326, XP 000564453 I. BENHAR ET AL. 'Mutations of two lysine residues in the CDR loops of a recombinant immunotoxin that reduce its sensitivity to chemical derivatization.' cited in the application see abstract see figure 1</p> <p>---</p>	1-30, 34-37
X	<p>CANCER RESEARCH, vol. 53, no. 2, 15 January 1993 PHILADELPHIA, PA, USA, pages 334-339, P. FRIEDMAN ET AL. 'BR96 sFv-PE40, a potent single-chain immunotoxin that selectively kills carcinoma cells.' see abstract see discussion</p> <p>---</p>	1-30
X	<p>THE JOURNAL OF IMMUNOLOGY, vol. 152, no. 5, 1 March 1994 BALTIMORE, MD, USA, pages 2377-2384, C. SIEGALL ET AL. 'In vitro and in vivo characterization of BR96 sFv-PE40.' see abstract</p> <p>---</p>	1-30
	<p>---</p> <p>-/--</p>	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 90, no. 16, 15 August 1993 WASHINGTON, DC, USA, pages 7538-7542, U. BRINKMANN ET AL. 'A recombinant immunotoxin containing a disulfide-stabilized Fv fragment.' cited in the application see abstract</p>	1-30
A	<p>NATURE, vol. 309, no. 5963, 3 May 1984 LONDON, GB, pages 73-76, H. KLOBECK ET AL. 'Contribution of human VkII germ-line genes to light-chain diversity.' cited in the application see figure 2</p>	1-22, 34-61, 65-88
A	<p>THE JOURNAL OF IMMUNOLOGY, vol. 150, no. 7, 1 April 1993 BALTIMORE, MD, USA, pages 2774-2782, U. BRINKMANN ET AL. 'Recombinant immunotoxins containing the VH or VL domain of monoclonal antibody B3 fused to Pseudomonas exotoxin.' see abstract</p>	1-30
A	<p>CANCER RESEARCH, vol. 54, no. 12, 1 July 1994 PHILADELPHIA, PA, USA, pages 3460-3467, M. CHOE ET AL. 'B3(Fab)-PE38M: A recombinant immunotoxin in which a mutant form of Pseudomonas exotoxin is fused to the Fab fragment of monoclonal antibody B3.' see abstract see figure 2</p>	1-30
P,X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 91, no. 25, 6 December 1994 WASHINGTON, DC, USA, pages 12051-12055, I. BENHAR ET AL. 'Rapid humanization of the Fv of monoclonal antibody B3 by using framework exchange of the recombinant immunotoxin B3(Fv)-PE38.' see abstract</p>	1-22, 65-88

-/--

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>PROTEIN ENGINEERING, vol. 7, no. 12, December 1994 OXFORD, GB, pages 1509-1515, XP 000482989 I. BENHAR ET AL. 'Cloning, expression and characterization of the Fv fragments of the anti-carbohydrate mAbs B1 and B5 as single-chain immunotoxins.' see abstract</p> <p>---</p>	1-22
P,X	<p>CLINICAL CANCER RESEARCH, vol. 1, no. 9, September 1995 PHILADELPHIA, PA, USA, pages 1023-1029, I. BENHAR ET AL. 'Characterization of B1(Fv)PE38 and B1(dsFv)PE38: Single-chain and disulfide-stabilized Fv immunotoxins with increased activity that cause complete remissions of established human carcinoma xenografts in nude mice.' see abstract</p> <p>---</p>	1-30
P,X	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 40, 6 October 1995 BALTIMORE, MD, USA, pages 23373-23380, I. BENHAR ET AL. 'Identification of residues that stabilize the single-chain Fv of monoclonal antibodies B3.' see the whole document</p> <p>-----</p>	1-30, 34-64

INTERNATIONAL SEARCH REPORT

ernational application No.

PCT/US95/13811

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 30,64,94
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 30,64 and 94 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0-A-9307286	15-04-93	AU-B- 2779892	03-05-93
		CA-A- 2120153	15-04-93
		EP-A- 0610286	17-08-94
		JP-T- 7502643	23-03-95
